SATURABLE BINDING OF OPIATE AGONISTS AND ANTAGONISTS TO SLICES OF RAT BRAIN TISSUE AND THE EFFECTS OF CHRONIC MORPHINE TREATMENT

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ABSTRACT

SATURABLE BINDING OF OPIATE AGONISTS AND ANTAGONISTS
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By

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The remarkable stereo- and chemical-specificity for the analgesia, tolerance and dependence induced by natural and synthetic narcotics led to the hypothesis of a specific narcotic receptor. In recent years *in vitro* ligand binding techniques have been employed in attempts to characterize this opiate receptor. In brain homogenates or partially purified membrane preparations saturable, stereospecific binding of narcotic drugs has been demonstrated. The binding affinity of opiate agonists and antagonists correlates quite well with their pharmacologic potency *in vivo*. These binding sites are enriched in areas responsive to morphine, particularly the periaqueductal and periventricular grey areas, which are widely held to be the site of narcotic-induced antinociceptive effects.

However, no alterations of this binding associated with the phenomenon of tolerance have been found after chronic morphine treatment. A receptor-mediated mechanism for tolerance is very strongly suggested by the parallel shift of the log dose-analgesic response relationship and by the insensitivity, after development of tolerance, of nerve cells in mammalian central nervous system to morphine-induced

suppression of firing. It is possible that the opiate binding sites available in homogenate and membrane preparations are not truly representative of the receptor *in vivo*. Therefore, the purpose of the present research has been to characterize saturable opiate binding to slices of brain tissue and determine the effects of chronic morphine treatment on this binding.

For these studies the striatum, diencephalon and anterior mesencephalon from freshly obtained rat brains were used. Thin slices or homogenates were prepared and used immediately. Tissues were incubated in a buffer solution with tritiated narcotic and other ligands and unbound drug removed by filtration. Two experimental designs were used to estimate binding constants: the saturation method (using increasing concentrations of radiolabelled drug) and the displacement method (using a fixed, low concentration of radiolabelled ligand and increasing concentrations of nonlabelled ligand).

The first group of experiments indicated that binding is different in the slice and homogenate preparations. In brain slices, the concentration of binding sites for ligand was greater and positive cooperativity among sites was observed. This activation was both homo- and heterotropic. Changes of opiate agonist binding associated with morphine tolerance were observed in the slice but not in homogenate preparations. Using both saturation and displacement methods, saturable and specific opiate agonist binding was found to be reduced following chronic morphine treatment. The reduction of specific binding reversed after withdrawal from drug.

Since sodium had been reported to reduce binding affinity of opiate agonists (in brain membrane preparations) it was hypothesized that the reduction of agonist binding affinity observed in slices

might be a manifestation of increased sensitivity to the effects of sodium ion. Although the differential effect of sodium ion to inhibit agonist and enhance antagonist binding was observed in the slice preparation this effect appears to be mediated by an alteration of agonist/antagonist accessibility to binding sites and of the degree of cooperativity among these sites. This is different from the changes observed with chronic morphine treatment. Furthermore, specific binding of naloxone was not greater in tolerant animals. Thus, although in superficial respects they are similar, it cannot be concluded that the effects of sodium and chronic morphine treatment are mediated by the same cellular perturbation.

The differences between the slice and homogenate preparations suggest important characteristics of the binding sites are not maintained after extensive cellular disruption. Since the alterations of binding associated with tolerance to morphine predicted by *in vivo* analgesic studies and *in situ* electrophysiological studies are observed in the slice preparation *in vitro* it is concluded that the binding site in slice more accurately represents the analgesic receptor *in vivo*.

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By

Mary Elizabeth Davis

A DISSERTATION

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to my parents, who believed

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INTRODUCTION

General Background

The pharmacologic effects of opium, from the juice of the poppy plant *Papaver somniferum*, have been appreciated for at least 50 centuries. The ancient Chinese used opium to treat dysentery, and opium derivatives are currently used for their constipating effects. The major uses of opiates at the present time involve their effects on the central nervous system. As an analgesic, morphine is particularly effective for relief of chronic, dull, visceral pain. The opiates appear primarily to alter the reaction to the painful stimulus rather than obtund the stimulus itself, and other sensory functions are not affected. Opiate derivatives are used, nontherapeutically, for their euphorigenic effects, and this property of the poppy plant was probably known to the Sumerians (ca. 5000 BC). Other important, but less desirable, effects of opiates are tolerance and physical dependence.

The mechanisms by which analgesia, tolerance and physical dependence are induced are not yet completely understood. The three appear to be closely related. Morphine and the synthetic opioids exist as stereoisomers. The (-)-forms are active while the (+)-forms are virtually inactive, incapable of inducing analgesia, tolerance or physical dependence. Some (-)-derivatives, with allyl or cyclopropyl substituents on the ring nitrogen, reverse the analgesic effects of opiates, prevent tolerance and precipitate withdrawal. The marked

chemical- and stereo-specificity for opiate analgesia led to the hypothesis of a specific tissue receptor which mediates the effects of opiates. Based on structure-activity relationships, the receptor has been proposed to have three points for attachment of opiates, corresponding to the cationic nitrogen of the piperidine ring, the phenol hydroxyl group and a hydrophobic surface. Interaction with the receptor and subsequent perturbation represents the molecular basis of drug action. Current research efforts to characterize this receptor include electrophysiological, biochemical and pharmacological studies. Although much progress has been made, the sequence of events by which analgesia is induced is still unknown. It is known that tolerance to opiates is not due to altered drug metabolism but to cellular alterations which result in a lower sensitivity of brain cells to opiate actions. Hypotheses to explain narcotic tolerance include immune mechanisms, redundancy of neural systems (with different sensitivities to opiates), alterations in synaptic transmission (noradrenergic, dopaminergic, cholinergic and serotoninergic systems have each been implicated) and alterations of the opiate receptor. The latter includes synthesis of inactive receptors (which compete with active receptors for opiate), reduction of receptor binding and failure of the receptor to transduce interaction with ligand to biologic effect. The overall purpose of this research has been to study the opiate receptor and alteration of its binding characteristics associated with narcotic tolerance.

Relationship Between Opiate Binding Sites and Analgesic Receptors

Characteristics of binding sites. *In vitro* studies have recently been employed in the characterization of an opiate binding site, and much evidence has been accumulated to support the hypothesis that this binding represents a pharmacologically relevant receptor for opiate agonists and antagonists. Like most drugs, opiates interact with tissue constituents other than their receptor by hydrophobic and ionic bonds. Since the hydrophobic and ionic characteristics are the same for the stereoisomers, these nonspecific interactions do not exhibit the marked stereospecificity of the narcotic analgesics and antagonists. Thus, Goldstein et al. (1971) were able to measure saturable, stereospecific binding of radiolabelled levorphanol as the difference between binding of radiolabelled levorphanol in the presence of 100-fold excess of its inactive stereoisomer dextrorphan and the presence of 100-fold excess of nonlabelled levorphanol. Using micromolar concentration of radiolabelled levorphanol, they found that stereospecific, saturable binding represents only 2 percent of the total binding, whereas nonsaturable and nonspecific binding account for 46 and 53 percent, respectively. With the availability of tritium-labelled opiate agonists and antagonists with much higher specific activity, Pert and Snyder (1973a), Simon et al. (1973) and Terenius (1973a) independently reported saturable, specific binding (that is, inhibited by excess of active opiate) of naloxone, etorphine and dihydromorphine, respectively, to crude homogenates and various subfractions of brain. In these studies the stereospecific binding was two to three times as great as nonspecific binding and followed the saturation kinetics expected for a drug-receptor interaction governed

by the law of mass action and involving a finite number of receptors. Stereospecific naloxone binding reaches equilibrium within ten minutes at 25° and 35°C and dissociation is very rapid (half time at 25°C is less than one minute). The binding is sensitive to high temperature and has a sharp pH optimum at 7.4 (C. Pert and Snyder, 1973b). Stereospecific etorphine binding is dependent on neither glycolysis nor oxidative metabolism, an indication that the binding site is not part of an active transport system (Simon et al., 1973). Accumulation of etorphine by brain slices was not inhibited by metabolic poisons (Huang and Takemori, 1976).

Relationship between potency *in vivo* and binding affinity *in vitro*. The specificity of this binding site has been studied extensively. Inhibition of stereospecific binding of ^{3}H-naloxone (C. Pert and Snyder, 1973a,b; C. Pert et al., 1976a), ^{3}H-etorphine (Simon et al., 1973) and ^{3}H-dihydromorphine (Wong and Horng, 1973) by a wide variety of non-labelled opiate agonists and antagonists, including benzomorphans, phenylpiperidines, diphenylamines, morphinans and oripavines, has been characterized. Log concentration versus percent inhibition curves are parallel, and the concentration to inhibit binding by 50 percent (IC50) compares favorably with their respective pharmacological potencies. These findings indicate that the various opiate agonists and antagonists interact with the same population of binding sites *in vitro*, and that the ability of these sites to bind opiates predicts potency *in vivo*. Other psychoactive, non-opiate drugs, such as phenobarbital, Δ9-tetrahydrocannibinol and caffeine and the non-narcotic analgesic aspirin, were inactive in ^{3}H-opiate binding inhibition studies. Similarly, no effect was found for the putative

neurotransmitters norepinephrine, dopamine, acetylcholine, γ-aminobutyric acid and serotonin, or for drugs which affect these systems (C. Pert and Snyder, 1973a,b; Terenius, 1973b; Wong and Horng, 1973). Creese and co-workers (1976) studied the structure-activity relationships of butyrophenones. They found that binding to the opiate receptor correlated with structural similarities of opiates, and not with neuroleptic activity. Thus, stereospecific binding to this site is limited to those substances which have narcotic analgesic agonist or antagonist actions. The affinity of the site *in vitro* directly correlates with pharmacologic potency and, for agonists, with inhibition of electrically-induced contractions of the longitudinal muscle of guinea pig ileum (Creese and Snyder, 1975).

Distribution of specific binding. Stereospecific opiate binding is found only in neural tissue (C. Pert and Snyder, 1973a), and phylogenetically is found in vertebrates but not in invertebrates (C. Pert et al., 1974a). Distribution of binding of ^{3}H-dihydromorphine (Kuhar et al., 1973) and ^{3}H-etorphine (Hiller et al., 1973) has been determined in fine detail using brains from monkey and human (obtained at autopsy), respectively. Receptor density is highest in the amygdala. Other portions of the limbic system, including the caudate nucleus, thalamic nuclei, hypothalamus, periaqueductal grey and periventricular grey, are rich in receptors. In analgesic studies, with intracranial microinjection of opiates, Jacquet and Lajtha (1973, 1974) and A. Pert and Yaksh (1975) have demonstrated that the periaqueductal and periventricular grey areas and medial hypothalamus are the locus of the antinociceptive effect of morphine. Opiate binding sites are

particularly abundant in these regions, suggesting that they represent the analgesic receptor. The regional distribution of the binding sites did not correlate with any neurotransmitter system. C.-Y. Lee and his co-workers (1975) characterized binding of ^{3}H-dihydromorphine and ^{3}H-naloxone using anatomically less discrete brain regions. For ^{3}H-dihydromorphine the striatum has the highest concentration of binding sites followed by midbrain, cerebral cortex, thalamus-hypothalamus-hippocampus and pons-medulla; the binding affinity in the various regions is similar but slightly greater in cerebral cortex. No ^{3}H-dihydromorphine binding to cerebellum was detected. The concentration of ^{3}H-naloxone binding sites varied much less (although greater in thalamus-hypothalamus-hippocampus), and differences of saturable ^{3}H-naloxone binding could be accounted for by regional variations of the affinity of the receptor for ^{3}H-naloxone. Kuhar et al. (1973) determined binding constants for unspecified brain areas and reported (but did not show) that regional differences of ^{3}H-dihydromorphine binding observed were due to variations in the number of binding sites rather than their affinity, in agreement with the results of C.-Y. Lee et al. (1975).

More recently, *in vivo* administration of potent, tritium-labelled antagonists naloxone or diprenorphine has been used to determine the distribution of opiate receptors. Specificity of binding was confirmed by inhibition of ^{3}H-drug binding *in vivo* by nonlabelled drug but not by the inactive enantiomorph. The number of binding sites per brain, concentration of drug required for half saturation of the sites, rate of dissociation and regional distribution for binding *in vivo* correlate well with previous results for binding *in vitro* (C. Pert and Snyder, 1975). After autoradiography opiate receptor grains were found

associated with structures of pathways for functions influenced by morphine, such as modulation of nociception (laminae I and II of spinal cord, periaqueductal grey, medial thalamus and nucleus raphe magnus) and coughing and vomiting reflexes (components of the vagal system including the dorsal motor nucleus of the vagus, nucleus tractus solitarius, nucleus ambiguus and nucleus commissuralis) (C. Pert et al., 1976b). Thus, stereo-specific opiate binding sites are found only in neural tissue and, among brain areas, binding site density is highest in those areas associated with functions altered by opiates. This, combined with the exquisite pharmacological and stereo-specificity of the binding site, supports the hypothesis that the binding site is the pharmacologic opiate receptor.

Endogenous ligand for the opiate receptor. The evidence which suggests that stereospecific opiate binding is the pharmacologic receptor does not lend itself to an explanation of why there would be such a receptor for a chemical of plant origin. Terenius and Wahlstrom (1974, 1975) reported a morphine-like substance in cerebrospinal fluid and in extracts of mammalian brains. Hughes (1975) first purified, characterized and, with his co-workers (Hughes et al., 1975), identified two pentapeptides with morphine-like properties, named enkephalins, from brain. Other endogenous morphinomimetic peptides identified include anodynin, from plasma (C. Pert et al., 1976c), pituitary opiate polypeptide (Teshemacher et al., 1975; Cox et al., 1975), various fragments of adrenocorticotrophic hormone (Terenius et al., 1975; Terenius, 1976) and fragments of β-lipotropin (Cox et al., 1976; Ling and Guillemin, 1976; Law et al., 1977). As a class, these peptides are referred to as endorphins (Simon, 1975).

The enkephalins have been studied most extensively. The primary structure of methionine-enkephalin is H-tyr-gly-gly-phe-met-OH; leucine is substituted for methionine in leucine-enkephalin (Hughes et al., 1975). The distribution of enkephalin (Hughes et al., 1975; Simantov et al., 1976b) and leucine-enkephalin-like immunoreactivity (Elde et al., 1976) is similar to that of opiate receptors and enkephalin is not found in liver, lung, cerebellum or guinea pig muscle (but is associated with the myenteric plexus). Methionine-enkephalin is highly folded with relative freedom around the N-terminal tyrosine-glycine residues and homologous to morphine (tyrosine corresponding to the phenol ring and terminal ammonium corresponding to the tertiary amine). Primary attachment may be by the tyrosine-glycine region and the relative freedom there could allow for secondary interaction (Jones et al., 1976; Roques et al., 1976).

Enkephalin has binding characteristics similar to those of opiate agonists (see below, Effects of Ions) (Pasternak et al., 1976; Simantov and Snyder, 1976a,b; Simantov et al., 1976a). Central administration of enkephalin induces short-lived analgesia; in rats rendered tolerant to morphine no analgesia was observed (Belluzzi et al., 1976; Buscher et al., 1976). The duration of analgesia may be the result of rapid degradation of enkephalin, as has been observed with brain homogenates (Hambrook et al., 1976). Cross tolerance between morphine and methionine-enkephalin was demonstrated using mouse vas deferens or guinea pig ileum from animals implanted with morphine pellets (Waterfield et al., 1976). Since enkephalin is endogenous it was felt that it might not induce dependence, and that related compounds with a longer duration might then be synthesized as narcotic-type analgesics devoid of dependence liability. Wei and Loh

(1976) demonstrated physical dependence to methionine-enkephalin or β-endorphin after infusion into either the periaqueductal grey or fourth ventricle.

Following chronic morphine treatment, the amount of enkephalin which can be extracted from brain tissue is increased. This was reversed by naloxone treatment one hour prior to sacrifice. This elevation of enkephalin activity was postulated to be indicative of decreased firing rate of "enkephalinergic" neurons, subsequent to feedback inhibition by morphine (Simantov and Snyder, 1976c). Decreased incorporation of ^{3}H-glycine into enkephalin and increased specific radioactivity of brain enkephalin fractions were found after chronic morphine treatment, whereas the opposite results (increased incorporation and decreased specific radioactivity) were found after precipitated withdrawal (Clouet and Ratner, 1976). These may indicate a decrease of enkephalin turnover during chronic morphine exposure and a rebound increase during withdrawal.

Methionine-enkephalin is found as residues 61-65 of β-lipotropin, a protein isolated from mammalian neurohypophysis (Li et al., 1965, 1976), and it has been suggested that β-lipotropin might be a precursor for methionine-enkephalin and possibly Goldstein's pituitary opiate polypeptide (Hughes et al., 1975). The fragment β-lipotropin 61-69, named β-endorphin, was found to have opioid activity in guinea pig ileum and opiate receptor binding assays while other fragments, including methionine-enkephalin, were less potent (Cox et al., 1976; Ling and Guillemin, 1976). β-lipotropin itself was inactive unless pre-incubated with a brain extract (Lazarus et al., 1976). Bloom and co-workers (1976) and Jacquet and Marks (1976) independently and simultaneously reported injections of β-endorphin, intracisternally

or into the periaqueductal grey, respectively, induced a naloxone-reversible catatonia-like state which was characterized by waxy flexibility. Both groups concluded that β-endorphin is implicated as a neuromodulator and that alterations of the β-endorphin system may underlie some psychopathological states.

The finding of an endogenous substance with activity similar to opiate agonists gives the opiate receptor physiological relevance (although now perhaps it would be better to refer to the enkephalin receptor). The physiology of the "enkephalinergic system" is presently widely studied. Endogenous opiate peptides have been implicated as endogenous releasing factors for growth hormone (Dupont et al., 1977) and prolactin (Lien et al., 1976). The endorphins have been claimed to mediate analgesia induced by stress (Akil et al., 1976a), electrical stimulation (Akil et al., 1976b,c), nitrous oxide (Berkowitz et al., 1976) and acupuncture (Pomeranz and Chiu, 1976). Others have noted an apparent lack of involvement of endogenous opiates in hypnotic anesthesia (Goldstein and Hilgard, 1975) and pain perception (El-Sobky et al., 1976; Goldstein et al., 1976).

Purification and Biophysical Properties of the Binding Site

Subcellular distribution. Purification and characterization of isolated opiate receptors has met with limited success; however, studies on the biochemical properties of nonpurified receptors have yielded interesting data. C. Pert and co-workers (C. Pert and Snyder, 1973a, 1976; C. Pert et al., 1974b) found that specific binding of dihydromorphine and naloxone was highest in synaptosomal

subfractions and, although binding was found in mitochondrial and microsomal fractions, they concluded that specific opiate receptor binding is localized on nerve terminals. Similar results were reported by Wong and Horng (1973). Based on sensitivity to tryptic digestion, Hitzemann and Loh (1974) concluded that stereospecific binding is on the extracellular surface of nerve ending particles. Smith and Loh (1976) studied the subcellular distribution of specific binding in detail. They reported both the microsomal fraction and purified synaptic plasma membranes contain stereospecific binding sites which could be distinguished only by their different densities (in sucrose gradients). The dissociation constants, inhibition of ^{3}H-naloxone binding by nonlabelled agonists and the effects of sodium ion and trypsin were similar for the two groups of binding sites. The possibility that the microsomal fraction was contaminated with synaptic plasma membrane was considered unlikely because the two fractions had similar amounts of binding. Separation of the microsomal fraction on a discontinuous sucrose density gradient showed the highest amount of binding to be in material less dense than synaptosomes. In autoradiographic studies specific opiate receptor grains were observed lying over as well as between cells (C. Pert et al., 1976b). Thus, opiate receptors are not limited to synaptic regions but rather are distributed diffusely on the surface of the cell. Such nonsynaptic receptors may be involved in modulation of neuronal function, rather than synaptic transmission.

Chemical composition. The opiate binding site in tissue homogenates and subfractions appears to be a proteolipid with a sulfhydryl group essential for specific binding. Sensitivity of opiate binding

to high temperature and nonphysiological pH suggested a protein component (C. Pert and Snyder, 1973b). Later it was shown that preincubating tissue preparations with proteolytic enzymes such as trypsin and chymotrypsin reduced specific binding (Hitzemann and Loh, 1974; Pasternak and Snyder, 1974, 1975a). Similarly, opiate binding is reduced by many detergents (Simon et al., 1975a) and phospholipase A (Pasternak and Snyder, 1974, 1975a), indicating a lipid moiety. Lowney et al. (1974) were able to partially purify stereospecific binding using proteolipid isolation techniques. Loh and co-workers (1974) noted the correspondence between apparent conformational requirements for the opiate receptor and the structure of cerebroside sulfate. Therefore, they studied the stereospecific binding of ^{3}H-etorphine and ^{3}H-naloxone to liposomes of commercially obtained cerebroside sulfate using the same techniques as Lowney et al. (1974). The results for proteolipid extracted from rat brain and commercial cerebroside sulfate were virtually identical, and cerebroside sulfate may be an important component of the receptor partially purified by Lowney et al. (1974). The analgesic potency and binding affinity to cerebroside sulfate correlated quite well for a series of opiate drugs. The analgesic potency was less if the amount of available cerebroside sulfate was reduced, either by spontaneous mutation (jimpy mice) or pretreatment with agents which bind to cerebroside sulfate (azure A or cetylpyridinum chloride) (Loh et al., 1975). In opiate binding studies azure A reduced the binding site concentration without affecting affinity for either morphine or naloxone. This effect was not observed with jimpy mutants; however, in these animals the binding site concentration was already reduced to the amount remaining after azure A treatment of normal brain

tissue (Law and Harris, 1977). Thus, cerebroside is strongly implicated as a functional portion of the opiate receptor.

Work in both Simon's (Simon et al., 1973; Simon and Groth, 1975) and Snyder's (Pasternak et al., 1975a; Wilson et al., 1975) laboratories has shown that a sulfhydryl group is essential for opiate receptor binding. Treatment of tissue preparations with reagents which alkylate sulfhydryl groups, such as N-ethylmaleimide, inhibits specific opiate binding. This inactivation could be prevented by allowing the tissue to bind either an opiate agonist or antagonist before alkylation, and the inactivation could be reversed by incubating the alkylated preparation with sulfhydryl-containing compounds. Protection afforded by ligand binding suggests that the critical sulfhydryl group is very close to the actual binding site or that binding causes a conformational change which makes the sulfhydryl group less accessible.

Effects of Ions on the Receptor *in vitro*

Sodium ion, at physiological concentration, has different effects on agonist and antagonist binding. Simon et al. (1973) first suggested that inhibition of ^{3}H-etorphine, but not ^{3}H-naloxone, binding by sodium might reflect a fundamental difference between opiate agonists and antagonists. This has been confirmed (C. Pert et al., 1973; C. Pert and Snyder, 1974; C.-Y. Lee et al., 1975; Simon et al., 1975a). Lithium ion has some discriminative effect, but potassium, rubidium and cesium are ineffective. More recently, Snyder's group has reported selective enhancement of ^{3}H-dihydromorphine binding by manganese, magnesium and nickel (Pasternak et al., 1975b), in contrast to their earlier reports of inhibition of both agonist and

antagonist binding by the same concentration of magnesium (C. Pert and Snyder, 1974).

Although initially there was disagreement, the bulk of the data indicates that the differential effect of sodium is primarily the result of a selective change of affinity for agonists and antagonists. Sodium ion is hypothesized to be an allosteric effector (C. Pert and Snyder, 1974; Simon and Groth, 1975; Simon et al., 1975b), which induces a change from a conformation with high affinity for agonist to one with high affinity for antagonist. Studies on receptor inactivation by sulfhydryl reagents provide further evidence for the hypothesis (Simon and Groth, 1975). As already noted, binding the receptor with either agonist or antagonist was protective against N-ethylmaleimide alkylation. The rate of inactivation is also decreased if the buffer contained 100mM sodium ion; lithium was slightly effective and potassium, rubidium and cesium were ineffective. Since ionic specificity for protection against N-ethylmaleimide and for alterations in binding affinity are the same, it was suggested that the same conformational change is involved in the two effects. Agonist binding was more sensitive than antagonist to degradation by sulfhydryl reagents (Pasternak et al., 1975a; Simon et al., 1975c; Wilson et al., 1975) and proteolytic enzymes (Pasternak and Snyder, 1975a).

The model of sodium ion as an allosteric effector predicts that the opiate receptor is oligomeric and that interactions among subunits (positive or negative cooperativity) should be observed in binding experiments. Thus far there is little evidence for such interactions. Saturable binding of ^{3}H-naloxone, etorphine and naltrexone has been reported to be hyperbolic and Scatchard or Klotz

plots of these, as well as ^{3}H-dihydromorphine binding, were considered linear, indicating independence of binding sites (C. Pert and Snyder, 1973b, 1974; Simon et al., 1973, 1975b; Wong and Horng, 1973; C.-Y. Lee et al., 1975). Simon et al. (1975c) noted that their allosteric effector model required interactions among subunits and concluded that deviations from linearity of their Scatchard plots indicated positive cooperativity. The Hill slope (an index of cooperativity) of their data was calculated to be 1.5 (Simon, 1976). Simantov and Snyder (1976b) found Hill slopes of 0.8 to 1.2 for opiate agonists and antagonists, as well as enkephalin. In the revised model proposed by Simon (1976) receptors exist primarily as dimers which interconvert between high agonist or antagonist affinity forms, with sodium as an allosteric effector. In low sodium concentration, the dimers dissociate into monomers which reassociate quite slowly and are much more sensitive to degradative treatments. The monomers cannot convert to the sodium dependent form (or do so very slowly), and thus account for the residual agonist binding observed at high sodium concentrations.

The differential effects of sodium on opiate receptor binding can also be accounted for by sodium's effect on membrane lipids. Narcotic agonist-cerebroside sulfate complexes are hydrophobic; that is, the agonist interacts with the macromolecule as an intimate ion pair. Conversely, antagonist-cerebroside sulfate complexes are hydrophilic and the binding is by a hydrated ion pair (Cho et al., 1976). Sodium makes the lipid more hydrophilic; thus, interaction with antagonists is favored (Loh, personal communication).

Electrophysiological Studies of Opiate Action

Microiontophoretic studies have provided evidence that binding of opiates to their receptor causes a change in sodium conductance. Zieglgansberger and Bayerl (1976) studied the mechanism of action of opiates using intra- and extra-cellular recording from single cells in laminae IV and V of the spinal cord. The dorsal horn neurons were responsive to noxious stimuli. Iontophoretically applied morphine reversibly depressed spontaneous, stimulus-evoked and L-glutamate-induced firing. This depression was blocked by naloxone. Systemic administration of morphine or fentanyl resulted in similar suppression. Detailed studies showed that microiontophoretically applied morphine did not hyperpolarize the cells or alter spike shape. The rate of rise of mono- and poly-synaptic excitatory postsynaptic potentials was decreased. Opiate inhibition of L-glutamate depolarization was due to postsynaptic blockade of increased membrane conductance. That naloxone antagonized only morphine inhibition of L-glutamate depolarization and not that of procaine, atropine or calcium argues for a specific opiate receptor. Microiontophoretically applied morphine depressed spontaneous discharge of cells in the sensorimotor cortex. The effect was reversed by naloxone (Satoh et al., 1976). Enkephalin applied to cells of the cerebral cortex decreased the spontaneous firing rate and inhibited L-glutamate-induced depolarization. Both effects were reversed by naloxone (Zieglgansberger et al., 1976). Naloxone reversible, enkephalin-induced depression of activity evoked by noxious stimuli has been found in areas implicated in opiate action, including thalamus and dorsal medulla (Hill et al., 1976) and caudate nucleus and periaqueductal grey (Fredrickson and Norris, 1976). *In situ*, narcotic

agonists block firing of nerve cells, including that firing induced by noxious stimuli. This block is reversible by naloxone and is due to decreased sodium conductance. Thus, narcotic agonists and antagonists differentially affect sodium permeability of neuronal membrane.

Effects of Morphine Treatment

Following chronic morphine treatment, sensitivity to the analgesic effects of morphine is reduced. Although a larger dose of morphine is required to induce the same degree of analgesia, a comparable maximal analgesic effect is observed if a sufficient dose is administered. This suggests that tolerance to the analgesic effects of opiates is mediated by a reduction of receptor affinity. Metabolism of opiate drugs is not enhanced following treatment with opiates. The depressant effects of microiontophoretically applied morphine and enkephalin on spontaneous and L-glutamate-induced firing were not observed in animals which had been chronically treated with morphine (Satoh et al., 1976; Zieglgansberger et al., 1976). This is strong evidence that tolerance to narcotics is receptor-mediated.

Attempts to correlate morphine tolerance with alterations of opiate binding have been, for the most part, disappointing. C.-Y. Lee et al. (1973) and Klee and Streaty (1974) observed no alterations of ^{3}H-dihydromorphine binding during development and offset of tolerance. Hitzemann et al. (1974) found some increases of saturable naloxone binding after various morphine, naloxone and/or placebo pellet treatment regimens. They concluded that there were neither qualitative nor quantitative effects on opiate binding associated

with chronic morphine treatment. C. Pert et al. (1973) reported that the number of receptor sites is increased by *in vivo* administration of a variety of opiate agonists and antagonists and concluded, based on the time course, that this increase is unrelated to tolerance or dependence. Further studies (C. Pert and Snyder, 1976) confirmed this. Treatment with morphine *in vivo* for five minutes was sufficient to maximally increase the number of ^{3}H-dihydromorphine binding sites. This increase was hypothesized to be the result of opiate displacement of enkephalin from receptors. While this is possible, it would result in a change in apparent affinity, not binding site concentration. More likely, their data indicate that binding *in vivo* protects the receptor from lytic enzymes released during homogenization, similar to the protection from degradation by sulfhydryl reagents noted earlier (Pasternak and Snyder, 1975a; Simon and Groth, 1975). Thus, in spite of evidence from microiontophoretic studies that tolerance to opiates is receptor-mediated, no alterations in receptor binding which correlate with tolerance have been observed.

Summary and Specific Objectives

Saturable, stereospecific binding of opiate agonists and antagonists has been widely studied. An endogenous ligand, enkephalin, with actions similar to opiate agonists and affinity for the opiate binding site has been purified from brain. The binding site appears to be a proteolipid localized on the extracellular surface and is found only in neural tissue. In brain the density of these sites is high in areas in which direct application of morphine induces analgesia and which are high in enkephalin content. The affinity of this

binding site for various opiate agonists and antagonists parallels their potency *in vivo*.

Binding studies of the opiate receptor have not convincingly demonstrated the interactions among ligand sites predicted by the allosteric model or alterations of binding concomitant with chronic morphine treatment. It is possible that cooperativity has not been observed because the capacity for interaction among subunits is not retained after homogenization, or that the experimental design was not optimal for demonstrating cooperativity. Similarly, alterations of binding induced by chronic morphine treatment may be lost or obscured during tissue preparation. The aim of this research was to characterize opiate binding in tissue slices and determine the effects of chronic morphine treatment on this binding. Three sets of experiments were performed.

In the first group of experiments the effect of homogenization on opiate binding to brain was studied, to establish whether binding is altered by homogenization. In binding studies, crude brain homogenates or partially purified membrane fractions are commonly used. The relevance of this binding to the *in vivo* situation is questionable. In addition to selecting for only certain populations of receptors, perturbation of the receptor and its environment during preparation of the tissue may alter binding. Alternatively, co-factors or activators required for binding may be lost during homogenization, or a substance which inhibits binding may be released. To assess this, binding of morphine and naloxone to either slices or crude homogenates of brain tissue was measured.

The purpose of the second group of experiments was to determine whether opiate binding sites in slices are differentially sensitive

to sodium and, if so, which binding constants are altered by sodium. It has been hypothesized that sodium acts by inducing a conformational change in the receptor, which might be reflected in the degree of cooperativity among sites. Therefore, the displacement method (which is more sensitive to cooperativity) was used in these experiments and binding of etorphine and naloxone to slices measured in the absence of sodium ion (Tris buffer) and in the presence of physiological concentration of sodium (artificial cerebrospinal fluid). In a separate series of experiments, sensitivity to sodium was assessed by measuring binding in different sodium concentrations.

The final group of experiments were designed to determine the effects of narcotic tolerance on opiate binding *in vitro*. As already noted, other investigators have found no changes of opiate binding to brain homogenates which could explain narcotic tolerance. However, my early experiments, using slices of brain, showed that saturable binding of morphine is reduced in tolerant rats. Therefore, further slice studies were conducted. In one series of experiments, parameters for morphine binding were determined, using the displacement method, during induction and offset of morphine tolerance. The degree of tolerance was assessed in similarly treated rats using warm plate and foot shock techniques. The effects of long term morphine treatment on morphine binding, *in vitro*, were also determined. The reduction of saturable morphine binding observed in morphine tolerant rats superficially resembles the effect of sodium ion on the opiate receptor. To test the hypothesis that the reduction of morphine binding, observed following chronic morphine treatment, is due to increased sensitivity to the effect of sodium on the binding sites, the effect

of sodium ion was assessed in tolerant rats, using the same protocols outlined above.

MATERIALS AND METHODS

Materials

Naloxone (tritium, randomly labelled) was purchased from New England Nuclear (Boston, MA) in ethanol solution. The specific radioactivity was 20 Curies/mmole and radiochemical purity was greater than 98%. Morphine (tritium, nominally labelled at C1) and etorphine (tritium, nominally labelled at C15 and/or C16) were purchased from Amersham/Searle (Arlington Heights, IL). Morphine was supplied in ethanol solution; the specific radioactivity was 27 and 30 Curies/mmole and radiochemical purity was greater than 96%. Etorphine was also supplied in ethanol; the specific radioactivity was 41 Curies/mmole and radiochemical purity was greater than 98%. In preliminary naloxone binding experiments, markedly different results were obtained with two different solutions of ^{3}H-naloxone. The two were compared with authentic naloxone, using thin layer chromatography in an appropriate solvent system. For one solution, the older one, there were two peaks of radioactivity, and the smaller of the two was associated with authentic naloxone. Therefore, during the subsequent course of binding experiments, the radiochemical purity of the tritiated drugs was routinely verified using thin layer chromatography. ^{3}H-Etorphine and morphine each decompose less than 2% per year, but ^{3}H-naloxone degrades in an autocatalytic fashion, and is stable for less than 6 months after opening. The tritium labelled drugs were used as supplied and were diluted daily, using

the appropriate buffer, from the purchased solution or a secondary dilution in ethanol.

Naloxone hydrochloride was purchased from Endo Laboratories (Garden City, NY) and morphine sulfate (U.S.P. powder) from Mallinkrodt Chemical Works (St. Louis, MO). Etorphine hydrochloride (M99) was the generous gift of Dr. J. H. Woods (Department of Pharmacology, University of Michigan). Other chemicals were reagent grade from commercial suppliers.

Methods

Binding protocol. Male, Sprague-Dawley rats (175-225 g) were decapitated and the brain rapidly removed. The brain tissue to be used in these studies (striatum, diencephalon and anterior mesencephalon) was dissected from the brain following the procedure of Nyback and Sedvall (1969). Briefly, the cerebellum was removed and cerebral cortex separated from the striatum and hippocampus, using the lateral ventricles as a guide. The hippocampus was removed, and meninges cleaned from the remaining brain stem-striatum. In early slice experiments slices were prepared freehand in the coronal plane; in the majority of experiments frontal slices, 0.5 mm thick, were made using a McIlwain tissue chopper. No slices were made posterior to the junction between anterior and posterior colliculi. All tissue preparation was performed in a cold room (temperature less than 5°C) and slices were kept in cold buffer on ice until used in the experiment (which was begun immediately after slices were prepared). Slices were incubated at 35°C for 20 minutes in a buffer solution with appropriate concentrations of tritiated and nonlabelled drugs and other ligands as indicated. Each slice was homogenized immediately after

the incubation. A Dounce-type tight fitting glass pestle was used in early experiments; a Potter-Elvehjem homogenizer with a motor driven Teflon pestle (type A, clearance 0.10-0.15 mm) was used in most of the experiments. Aliquots (0.5 ml) were passed on nitrocellulose filters (0.8 μm pore size, Millipore Corp., Bedford, MA) and rinsed with 10 ml of ice cold buffer, to separate unbound from bound ^{3}H-labelled drug. In this protocol homogenization is used so ^{3}H-labelled drug which may be transported and dissolved in cytoplasm, but not bound to any particular cellular constituent, can be removed during filtration. In some experiments, the slice incubation was performed in duplicate, while in others duplicate aliquots of a homogenate were filtered and assayed separately. To determine the effects of homogenization on binding, results from slice experiments were compared with those from crude tissue homogenates. For the latter, the striatum and caudal diencephalon were dissected as above, and homogenized using the same type of homogenizer and approximately equal protein concentration, as in the analogous slice experiment. Otherwise, incubation and separation of unbound drug by filtration were the same as in the slice experiments.

Filters were dissolved in 1 ml ethylene glycol monomethyl ether ("Piersolve", Pierce Chemical Co.) and 10 ml of counting solution (4 g 2,5-diphenyloxazole (PPO), 167 mg 1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]-benzene (dimethyl-POPOP), 250 ml Piersolve and toluene to make 1 liter) added. Radioactivity was measured at 27% efficiency using a Beckman LS 100 liquid scintillation counter, or at 35% efficiency using a Searle Analytic Isocap 300 liquid scintillation counter. The external standard channel ratio was used to monitor uniformity of quench and the internal standard method used to determine

efficiency. Protein concentration was determined in duplicate by the method of Lowry et al. (1951) on diluted samples or by the biuret method (Layne, 1957). Standards were prepared from bovine serum albumin (crystallized, or fraction V, Sigma Chemical Co.) and the same buffer used as in the tissue samples to be assayed.

Artificial cerebrospinal fluid (CSF) or 50 mM Tris-HCl buffer (pH 7.4) were used as incubation media. The same buffer was used in tissue preparations and drug dilutions. Both were made with double distilled water. The composition of CSF was 125 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 25 mM $NaHCO_3$; the solution was bubbled with 95% O_2-5% CO_2 for one hour prior to use (Davson, 1967).

Saturable binding was determined as the difference between ^{3}H-labelled drug bound in the absence (i.e., total binding) and presence (i.e., nonsaturable binding) of 10^{-5} M nonlabelled drug. Five concentrations of ^{3}H-drug were used for each experiment. Dissociation constants and binding site concentrations were estimated using Scatchard analysis of the saturable binding data (Scatchard, 1949). Linear least squares regression was used to determine the line of best fit to the data (Sokal and Rohlf, 1969).

In displacement experiments a fixed, low (1-3 nM) concentration of ^{3}H-drug was used and nonlabelled drug concentration was varied from less than to greater than thirty times the concentration of ^{3}H-drug. Nonsaturable binding was determined concurrently as ^{3}H-drug bound in the presence of 10^{-5} M nonlabelled drug. In these experiments the difference between ^{3}H-drug bound (at any concentration of nonlabelled drug) and nonsaturable ^{3}H-drug bound is termed specific binding. Dissociation constants, binding site concentrations, and

indices of cooperativity were determined directly from the displacement data by the analysis described below.

Theory of displacement analysis. Binding to a receptor follows the law of mass action and is described by the Hill equation as:

$$b = \frac{B_{max} \quad [D]^n}{K_D^n + [D]^n} \tag{1}$$

where b is the amount of drug bound at the concentration D, K_D is the dissociation constant (concentration of drug required for half maximal binding), B_{max} is the amount of drug bound at infinitely high drug concentration and thus is the concentration of binding sites, and n is the number of interacting sites. For the case of n=1 a plot of b vs [D] will describe an adsorption isotherm (Figure 1, solid line). If n is not equal to one deviations from hyperbolicity occur. As n increases above one (positive cooperativity among sites) this curve becomes sigmoidal (Figure 1, dashed and broken lines) and the shape of the curve is determined by the magnitude of n. Scatchard plots are linear only if n is one; if n is greater than one they curve characteristically (Figure 2). Thus, the existence of positive cooperativity can be inferred from this analysis but the degree of interaction cannot be assessed. Furthermore, neither the binding site concentration nor dissociation constant can be estimated if plots are nonlinear. Thus, it is inappropriate to use these types of analyses for the opiate receptor, in which positive cooperativity is predicted by the model. Instead, a plot of the data as $\log[b/(B_{max}-b)]$ vs $\log[D]$, the Hill plot, is generally used to analyze such data. This function is linear with a slope of n and intercept $-n \log K_D$ (see Segel, 1975). This analysis, however,

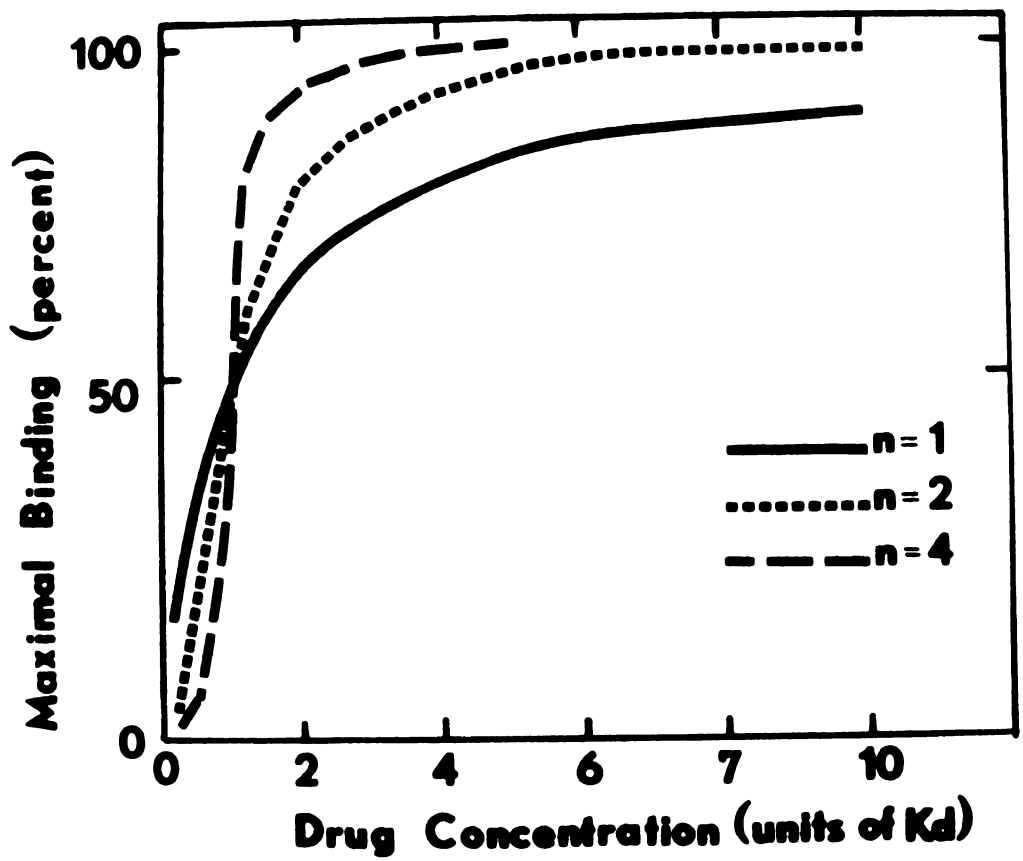


Figure 1. Theoretical curves for drug-receptor binding determined by the saturation method. Calculation was based on equation 1, with B_{max} and K_D held constant, and n (number of interacting sites) equal to 1, 2 and 4.

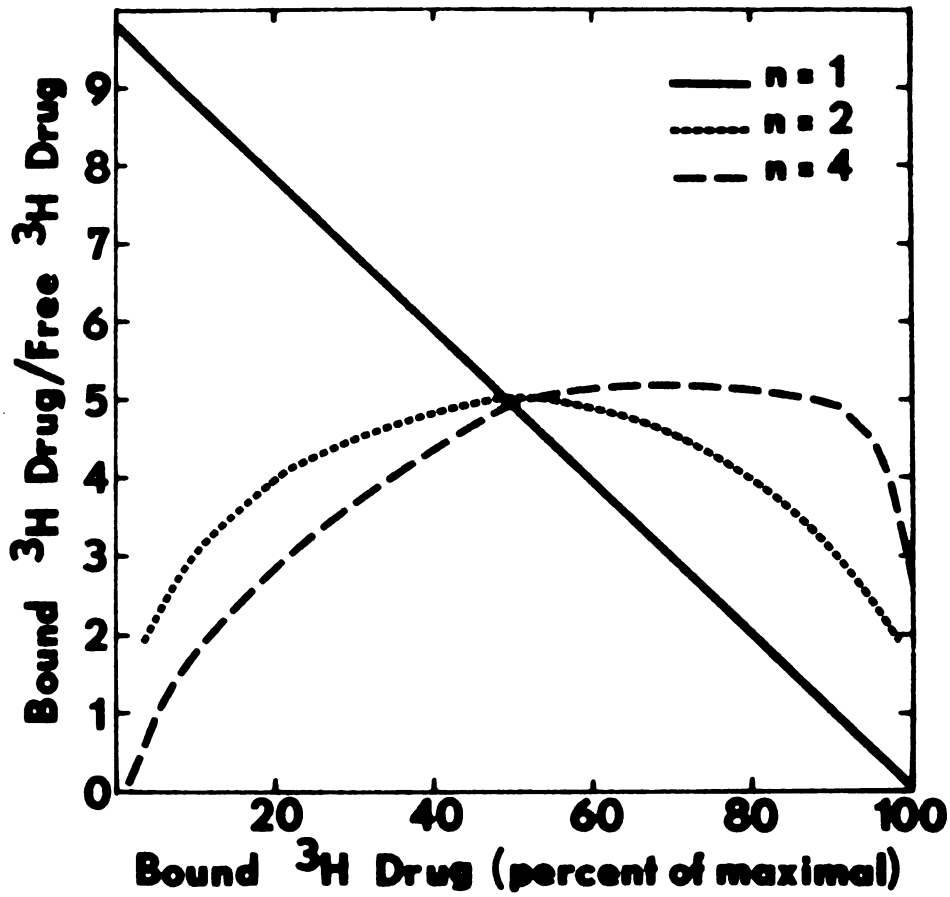


Figure 2. Scatchard plot of theoretical drug-receptor binding curves. Theoretical data from Figure 1 were calculated for Scatchard analysis.

requires an accurate determination of B_{max}, which is difficult to do because at high concentrations of ^{3}H-drug, approaching saturation, the saturable binding is a small fraction of the total binding. Additionally, values obtained from a Hill plot under these conditions underestimate the magnitude of n (Endrenyi et al., 1975).

Binding of ^{3}H-drug in the displacement experiment (b_1) is given by the function:

$$b_1 = \frac{B_{max} \quad (d+a)^n}{K_D{}^n + (d+a)^n} \times \frac{d}{d+a} + C \qquad (2)$$

where d is the concentration of radiolabelled drug, a is the concentration of nonlabelled drug, and C is the nonsaturable binding. If n is one, this function is essentially an isotope dilution curve (Figure 3, solid line). However, if binding is cooperative (n greater than one) and the concentration of labelled ligand less than the K_D, the amount of ^{3}H-drug bound increases initially, at relatively low concentrations of nonlabelled drug, and then decreases (Figure 3, dashed and broken lines). The binding constants B_{max}, K_D, n and C for the function (equation 2) which best represent the experimental data were found using Brown's method to solve the normal equations (Brown, 1969) and/or the Marquardt algorithm (Marquardt, 1963) for minimizing a sum of squares of nonlinear functions. A CDC 6500 computer was used in the analyses.

Chronic morphine treatment. Animals were treated with morphine by pellet implantation. Pellets containing 75 mg of morphine base (formulated according to Way et al., 1969) were implanted in the dorsal subcutaneous space. To study the effects of morphine tolerance, animals were implanted with two pellets, the second 36 hours after

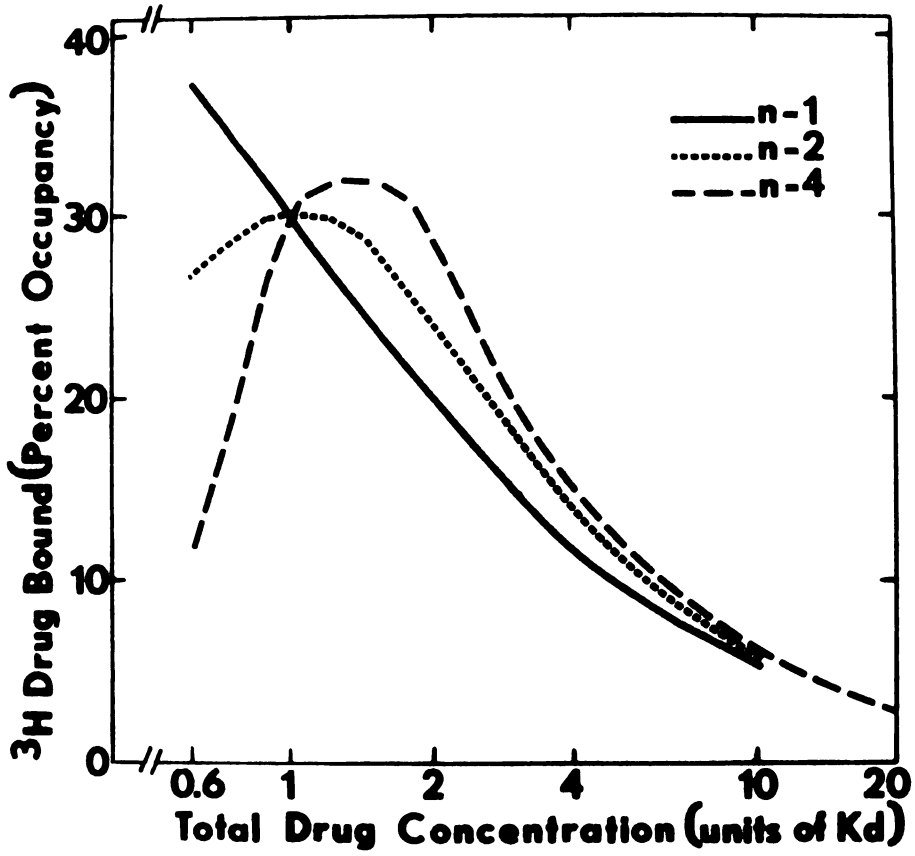


Figure 3. Theoretical curves for drug-receptor binding determined by the displacement method. Calculation was based on equation 2, with B_{max}, K_D and n the same as in Figure 1.

the first, and sacrificed 72 hours after implantation of the first pellet. For the time course experiments, the 36 hour morphine treatment group received only one pellet. Withdrawal was initiated after 72 hour morphine treatment by removing both pellets and animals used 1, 4 or 7 days later. Methoxyflurane anesthesia was used for all surgical procedures.

For three week morphine administration, animals were implanted with subcutaneous cannulae made of polyethylene tubing. The rats were maintained in a harness connected to a swivel by a tightly coiled spring which allowed the rats freedom of movement within the cage (Weeks, 1972; Brown et al., 1976). The dose of morphine sulfate on the first day was 18 mg/kg; this was gradually increased to 210 mg/kg/day over one week. During this time the drug was administered by constant infusion (0.0018 ml/min, with an infusion pump). Animals were maintained at the highest dose by injection (10 µl/100 g body weight) four times per hour using a pneumatically driven microliter syringe (Weeks, in preparation).

Assessment of analgesia. Tolerance to morphine was quantified by determining the morphine log dose-response relationship in sham operated, morphine treated (72 hour pellet implantation) and withdrawn (1, 4 and 7 days) rats. Analgesia was measured using warm plate (O'Callaghan and Holtzman, 1975) or electric foot shock (Tilson et al., 1973) procedures. Each rat was used only once and morphine treated rats were withdrawn for six hours prior to test. For the warm plate test, a hot plate thermostatically controlled at 50°C (Technilab Instruments) was used and the end point was licking of the hind paws. Before the test session, rats were placed individually in

the test apparatus (11" x 11" surface with a 5 1/4" high plexiglass enclosure) for 2 minutes for habituation. The control reaction time was determined and appropriate dose of morphine sulfate administered subcutaneously. Rats were tested 15 minutes after injection, and the difference between test and control reaction times recorded. Each rat was allowed 120 seconds above its control time, and rats not reacting within this time were given a score of 120. A plexiglass chamber (6 3/4" x 8 1/4" x 14") with a grid floor was used for foot shock test. Electric current was passed through the grids by means of a constant current shock scrambler (Lafayette Instruments) with a variable resistor which the experimenter could use to change the shock intensity. Initial intensity was 0.5 mA and this was increased in 0.02-0.05 mA increments. A movement of the forepaws associated with audible vocalization, elicited by the same shock intensity on 3 trials, was the endpoint. Shock was presented only when all four paws were on the grid and presentations were given at intervals greater than 10 seconds. The challenge dose of morphine was administered and shock threshold determined 15 minutes later. Results were expressed as percent increase above the control reaction (before administration of the challenge dose of morphine).

Data were analyzed by simple linear regression using an Olivetti Programma 101 microcomputer. Parallelism of the regression lines was tested and confidence intervals for the dose required for a change of 60 seconds on warm plate test or a 60% increase on the foot shock test was determined for each group (Sokal and Rohlf, 1969). The level of significance chosen was $p<.05$.

RESULTS

Effects of Homogenization

To determine the effects of homogenization on opiate binding sites, binding of tritiated ligands to slice and homogenate preparations of brain tissue was compared. Tris buffer was used in these experiments. Initial saturation experiments revealed differences between the two tissue preparations. In these experiments, tissue was incubated with increasing concentrations of ^{3}H-morphine (Figure 4), and at each concentration the amount of saturably bound ^{3}H-morphine was estimated as the binding of ^{3}H-morphine which could be displaced by excess nonlabelled morphine (the difference between total and nonsaturable binding in Figure 4). The total amount of ^{3}H-morphine bound (in the absence of nonlabelled morphine) was greater in slice than homogenate (Figure 4), while the amount of nonsaturable binding (in the presence of excess nonlabelled morphine) was the same in the two preparations. Saturable binding was greater in the slice than homogenate preparation. In brain homogenates, the saturable binding curve was hyperbolic, over the concentration range studied; however, in brain slices, some deviation, from the theoretical hyperbolic curve predicted from saturation kinetics, was found at the lowest concentrations. For the slice experiments, saturable binding varied from 65% (at 1 nM ^{3}H-morphine) to 35% (at 40 nM ^{3}H-morphine) of total binding; for brain homogenates saturable binding varied between 40 and 11% of total binding. The concentration

Figure 4. Binding of ^{3}H-morphine determined by the saturation method.

Panels A and B, initial experiments using slice and homogenate preparations. * indicates different from respective value for homogenate ($p<.05$). Panel C, slice experiment was repeated using modified protocol. Total and nonsaturable binding of ^{3}H-morphine were determined in the absence and presence of 10^{-5} M nonlabelled morphine, respectively. Saturable binding was calculated for each experiment as the difference between total and nonsaturable binding.

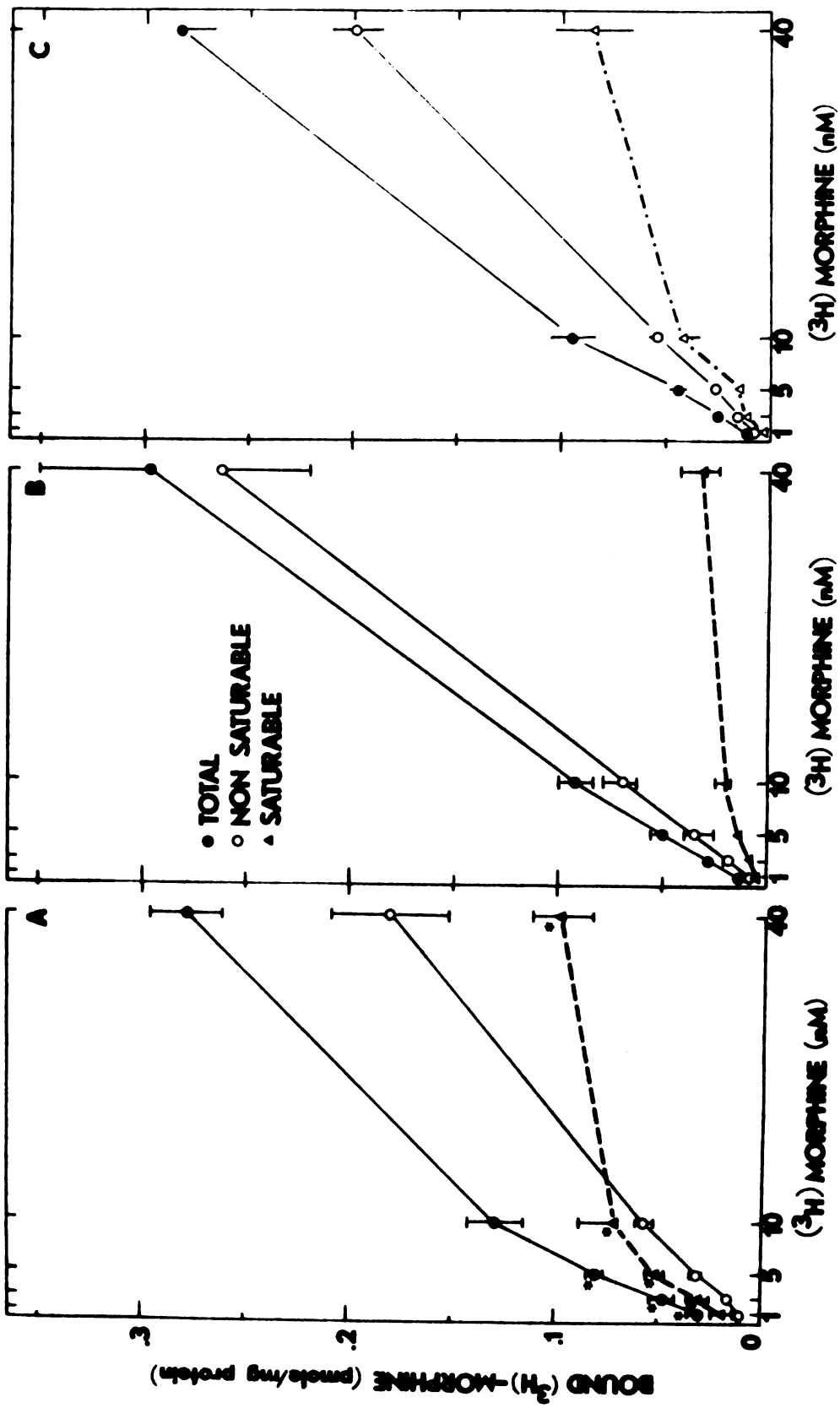

A
B
C
• TOTAL
○ NON SATURABLE
▲ SATURABLE
BOUND (^{3}H)-MORPHINE (pmole/mg protein)
(^{3}H) MORPHINE (nM)
0
.1
.2
.3
1
5
10
40

Figure 4

of binding sites (B_{max}) and dissociation constants (K_D's, the concentration of ligand necessary to bind half the sites, also the reciprocal of the binding affinity) were estimated from the saturable binding data using Scatchard analysis (Table 1). The binding site concentration was approximately three times greater in brain slices than homogenates, and these sites had greater affinity for ^{3}H-morphine (K_D was less in brain slices). Because the saturable binding curve was not hyperbolic, the binding constants for brain slices can only be considered rough estimates.

The nonhyperbolicity of saturable binding curves, in the slice preparation, could be the result of interactions among the binding sites. Therefore, slice and homogenate preparations were compared again, using the displacement protocol (which is not invalidated by interactions among sites). Positive cooperativity among binding sites was observed in brain slices, but not homogenates. When brain homogenates were used, the nonlabelled drug inhibited binding of ^{3}H-drug in a concentration-dependent fashion (Figures 5 and 6), similar to an isotope dilution curve. With brain slices, however, the nonlabelled drug initially enhanced the binding of ^{3}H-drug and then, at higher concentrations, inhibited the binding of ^{3}H-drug. This initial increase of ^{3}H-drug binding, at relatively low concentrations of nonlabelled drug, is indicative of positive cooperativity, and can occur only if the index of cooperativity is greater than one and the concentration of ^{3}H-drug is less than the dissociation constant. The magnitude of the initial increase is related to the index of cooperativity and dissociation constant; therefore, comparisons between drugs or treatments should be based on values determined from regression analysis (see below).

TABLE 1

Morphine Binding Constants for Brain Slices and Homogenates*

	K_D (nM)	B_{max} $\frac{\text{fmoles}}{\text{mg protein}}$
Homogenate	9.7	42
Slice (initial)	6.6	120
Slice (modified protocol)	19.4	106

*Estimated from Scatchard plots of saturable morphine binding data shown in Figure 4. The line of best fit was determined by linear regression.

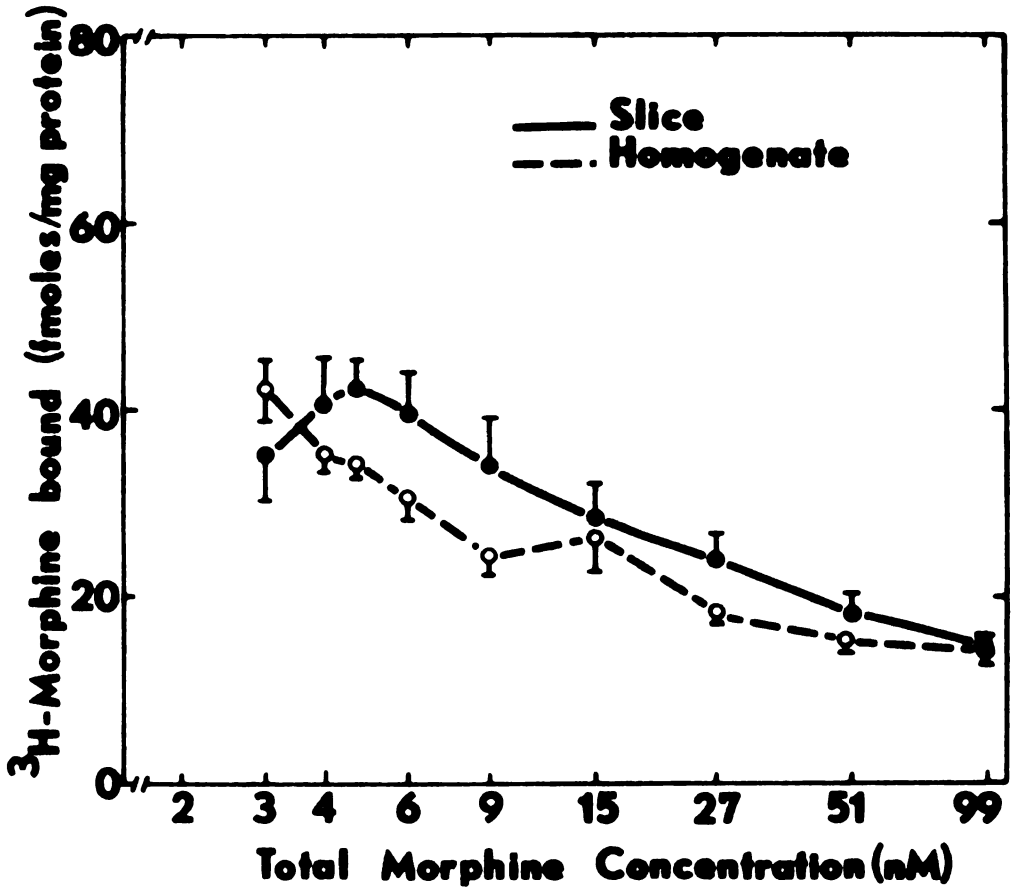


Figure 5. Comparison of ^{3}H-morphine binding in slice and homogenate preparations.

The displacement method was used. Binding of 3 nM ^{3}H-morphine was determined at various concentrations of nonlabelled morphine; the total concentration of morphine is shown on the abscissa. Values are shown as mean $\pm$ S.E.M. of 5 experiments for the slice preparation and 3 experiments for homogenate.

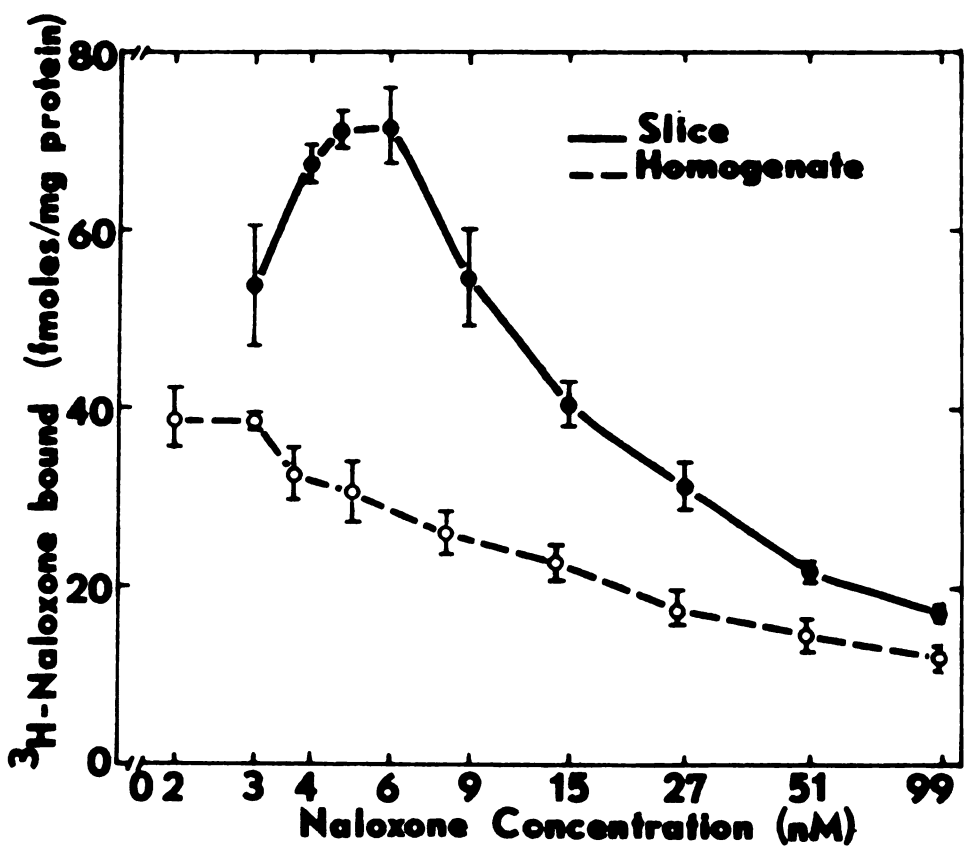


Figure 6. Comparison of ^{3}H-nalaxone binding in slice and homogenate preparations.

The displacement method was used. Values are shown as mean ± S.E.M. of 4 experiments for slice and 3 for homogenate. ^{3}H-naloxone concentration of 3 nM in slice and 2 nM in homogenate.

For displacement experiments using brain homogenate, the initial increase of ^{3}H-ligand binding, at low concentrations of nonlabelled ligand, was not observed for either morphine (Figure 5) or naloxone (Figure 6). The concentration of ^{3}H-morphine used (3 nM) was less than the K_D estimated from saturation studies (9.7 nM); therefore, the concentration of ^{3}H-drug was sufficiently low to detect cooperativity, if present. Direct analysis of the displacement data was not possible due to the shallowness of the curve (in this case small alterations of observed binding cause large changes in K_D or B_{max}). Although using larger concentrations of tritiated ligand would have given a steeper curve, the effect of interacting sites would have been much less pronounced. Thus, it can be concluded that cooperativity is either nonexistent or greatly attenuated if the tissue is homogenized prior to the binding incubation.

The binding constants for slice experiments were determined directly from the displacement data (Table 2). For both ligands, the minimum number of interacting sites was around 3, indicating strong, positive cooperativity among sites. In order to demonstrate the cooperativity of binding with more conventional methods, the displacement data were converted into saturation type data, and then calculated for Hill plot analysis (Figure 7). The slope of the line (n_H) was greater than 1 for both morphine (n_H = 2.6) and naloxone (n_H = 3.4). The values for n, determined by the two methods, are in good agreement.

The differences in results obtained with homogenate and slice preparations indicate that the opiate binding site, *in vitro*, is affected by homogenization. *In vitro* binding to the brain slice preparation appears to be a better model, since normal relationships

TABLE 2

Binding Parameters* for Opiate Binding Sites in Brain Slice

	B_{max} fmoles / mg protein	K_D (nM)	n	C fmoles / mg protein
Morphine	64	3.8	3.1	14
Naloxone	132	3.8	3.6	14

*Binding parameters were determined by nonlinear least squares regression analysis of all data points to equation 2, using Brown's method to solve the normal equations and/or the Marquardt algorithm for minimizing a sum of squares of nonlinear functions. A CDC 6500 computer was used in the analysis. B_{max} is the binding site concentration, K_D (dissociation constant) the concentration of drug at which half of the sites are bound, n is the number of interacting sites, and C is the nonsaturable binding. Binding was assayed in the absence of sodium.

Figure 7. Hill plot.

Data from naloxone and morphine displacement experiments using the slice preparation were converted into saturation type data and then calculated for Hill plot analysis. Linear least squares regression was used to determine the line of best fit. The slope of the line gives the number of interacting sites.

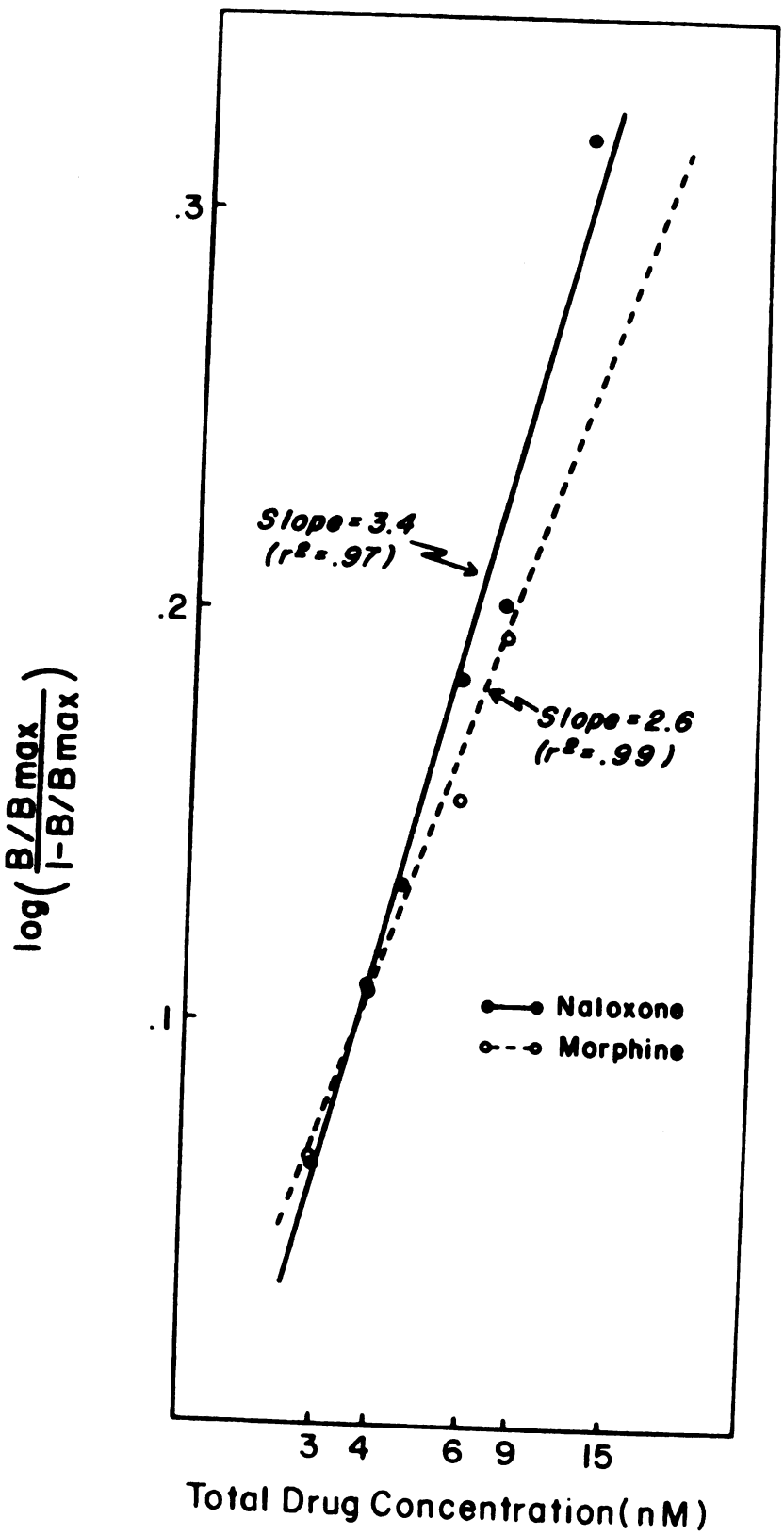

.3
.2
.1
Slope = 3.4
(r² = .97)
Slope = 2.6
(r² = .99)
Naloxone
Morphine
3 4 6 9 15
Total Drug Concentration (nM)
log(B/Bmax / 1-B/Bmax)

Figure 7

between receptor and cell are maintained, and thus binding to brain slices would be more relevant physiologically and pharmacologically. Compared to brain homogenate, both binding site concentration and affinity are greater in the slice preparation. Furthermore, in brain slices positive cooperativity among binding sites was observed. While this cooperativity is expected from the allosteric model of the opiate receptor, such interaction among binding sites was not observed in the homogenate, in the present study, confirming earlier studies by other investigators (C. Pert and Snyder, 1973b; Simantov and Snyder, 1976b).

Effect of Sodium on Opiate Binding Sites *in vitro*

Etorphine and naloxone binding in Tris buffer and artificial CSF. The opiate binding site is apparently located on the extracellular surface of the cell (Hitzemann et al., 1975) and thus is normally bathed in cerebrospinal fluid (CSF). Incubating the brain slices in artificial cerebrospinal fluid would be more physiological, and therefore more relevant, than in a buffer such as Tris. Results from homogenate experiments have shown a differential effect of physiological concentration of sodium ion on agonist and antagonist binding (C. Pert and Snyder, 1974; Simon et al., 1975b; C.-Y. Lee et al., 1975). Therefore, binding to brain slices of an agonist and antagonist was compared in Tris buffer and artificial CSF. Preliminary experiments indicated that morphine binding is greatly inhibited in artificial CSF (see Figure 12, 150 mM sodium), so the potent narcotic agonist, etorphine, was used.

Saturation studies of ^{3}H-etorphine binding in Tris buffer demonstrated the drawbacks of this method (Figure 8). The saturable binding did not appear to saturate and, at 2.5 nM ^{3}H-etorphine, the variability around the saturable binding was greater than 16% (with 5 experiments). Since the nonsaturable binding increases linearly, with increasing concentration of ^{3}H-etorphine, but the saturable binding increases less than proportionally with increasing concentrations of ^{3}H-etorphine (approaching the maximal, saturable binding asymptotically), the saturable binding becomes an increasingly small fraction of the total binding, and thus variability increases. With this ligand, the displacement method is more appropriate, even if there is no cooperativity, because the nonsaturable ^{3}H-drug binding remains constant. Similar results were found with ^{3}H-naloxone (Figure 9). Differences in binding of both etorphine and naloxone in Tris buffer and artificial CSF were more apparent at the higher ligand concentrations; however, these differences were obscured by the greater variability.

Nonsaturable binding of ^{3}H-etorphine was larger in CSF than Tris buffer. This was also observed for ^{3}H-naloxone, and has been reported for naloxone and dihydromorphine (C.-Y. Lee et al., 1975). Therefore, to compare binding in Tris buffer and CSF, results of displacement experiments were expressed as specific binding of ^{3}H-drug. Specific binding was calculated as the difference between ^{3}H-drug bound at nanomolar concentrations of nonlabelled drug and in the presence of excess (10^{-5}M) nonlabelled drug (which was determined for each experiment).

At the lowest concentrations of nonlabelled etorphine, specific binding of ^{3}H-etorphine tended to be lower in CSF than Tris buffer (Figure 10). The dissociation constant was not different in the two

Figure 8. Binding of ^{3}H-etorphine to brain slices determined by saturation method.

Binding of ^{3}H-etorphine was measured in tris buffer and artificial CSF using the saturation method. Saturable binding was calculated as the difference of ^{3}H-etorphine binding in the presence (nonsaturable) and absence (total binding) of 10^{-6} M unlabelled etorphine-HCl. Values are shown as the mean $\pm$ S.E.M. (unless within the space occupied by the symbols) of 5 (Tris) or 3 (CSF) experiments.

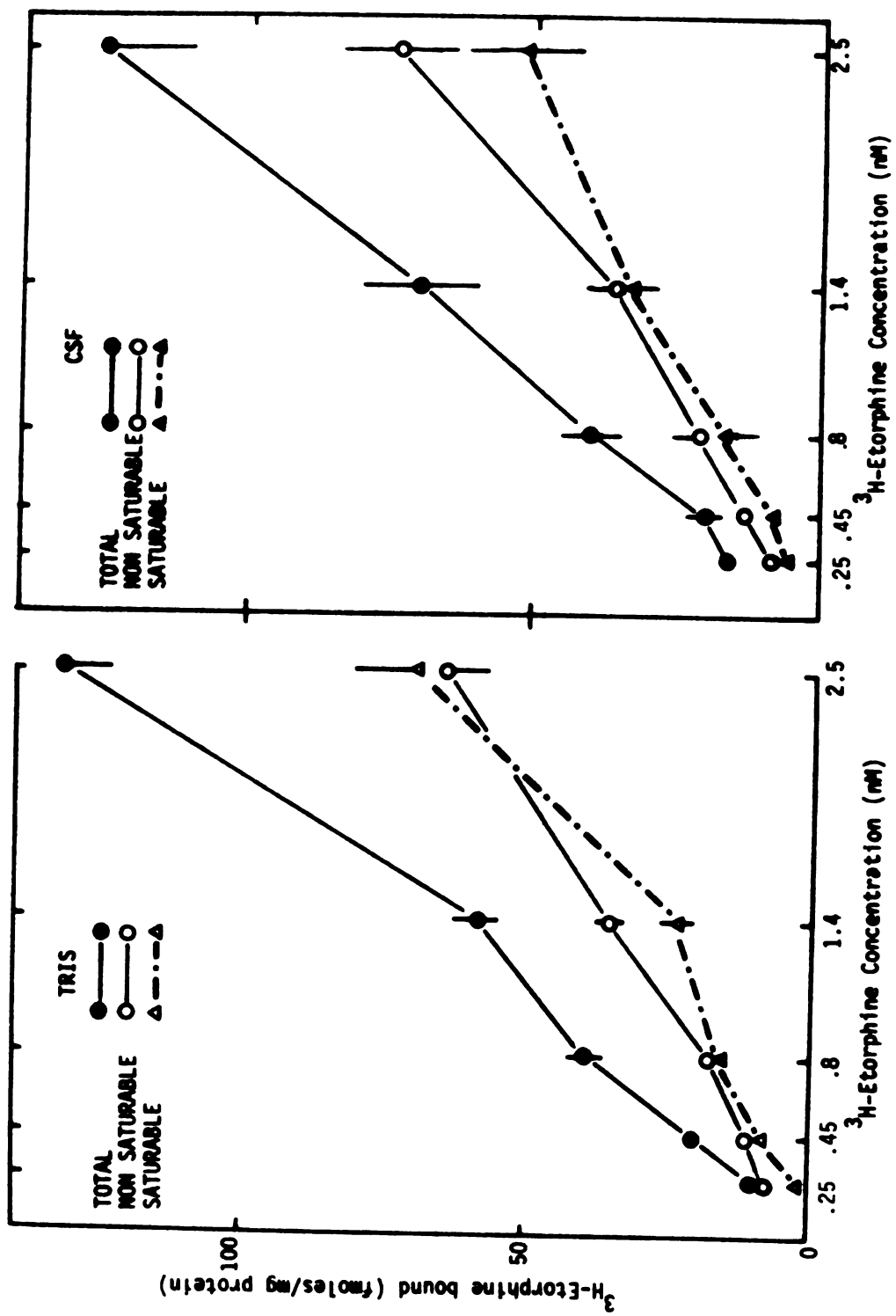

TRIS
TOTAL
NON SATURABLE
SATURABLE
CSF
TOTAL
NON SATURABLE
SATURABLE
^{3}H-Etorphine bound (fmoles/mg protein)
100
50
0
.25
.45
.8
1.4
2.5
^{3}H-Etorphine Concentration (nM)

Figure 8

Figure 9. Binding of ^{3}H-naloxone to brain slices, determined by saturation method.

Binding of ^{3}H-naloxone was measured in Tris buffer and artificial CSF. Saturable ^{3}H-naloxone binding was calculated as the difference of binding in the presence (nonsaturable) and absence (total binding) of 10^{-5} M naloxone-HCl. Values are shown as mean $\pm$ S.E.M. (unless within the space occupied by the symbol) of 4-6 experiments.

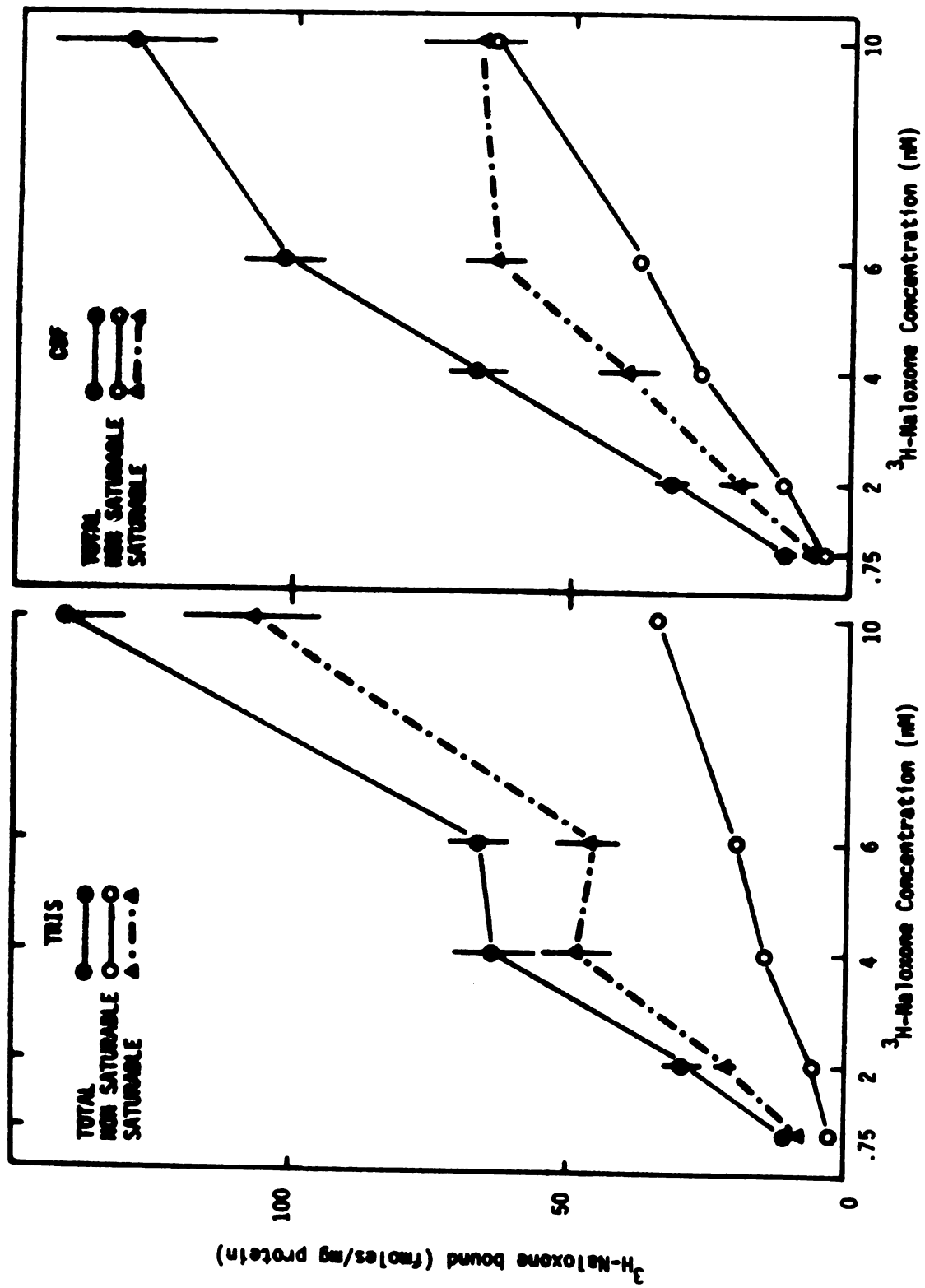

TRIS
TOTAL
NON SATURABLE
SATURABLE
CSF
TOTAL
NON SATURABLE
SATURABLE
^{3}H-Naloxone bound (fmoles/mg protein)
100
50
0
.75
2
4
6
10
^{3}H-Naloxone Concentration (nM)

Figure 9

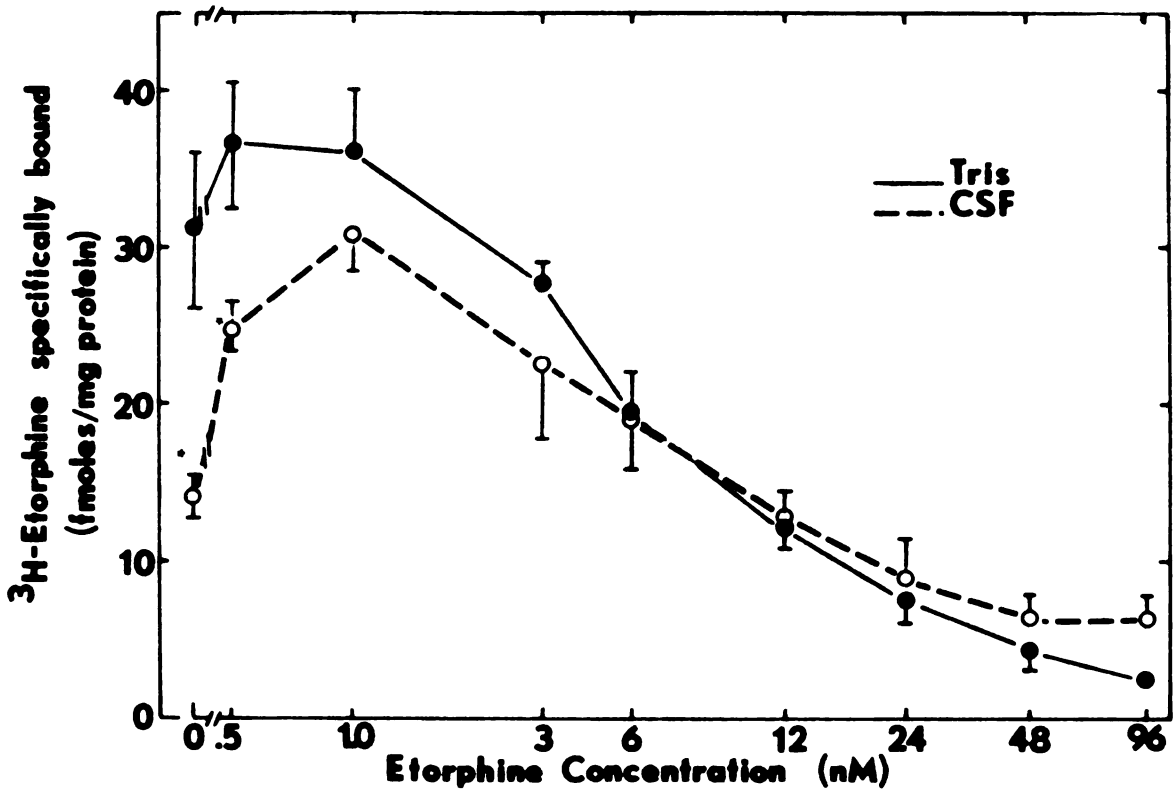


Figure 10. Binding of ^{3}H-etorphine to brain slices.

Binding of ^{3}H-etorphine (1 nM) was measured in Tris buffer and CSF, using the displacement method. The concentration of nonlabelled etorphine is shown on the abscissa. Nonsaturable binding (not shown) was determined in the presence of 10^{-5} M nonlabelled etorphine. Data are shown as mean $\pm$ S.E.M. of 5 experiments for Tris and 4 for Tris. Binding constants determined for the data are shown in Table 3.

solutions (Table 3). In this instance, then, the greater magnitude of the initial stimulation of ^{3}H-etorphine binding (by nonlabelled etorphine) is indicative of an increase in the interaction among sites, and this was found for the value of n_{app}, estimated by regression analysis (Table 3). The concentration of binding sites was less in CSF. These findings may explain the results of saturation studies (Figure 8), in which the "saturable" binding did not appear to saturate. In Tris buffer the saturable binding was initially more hyperbolic and reached a greater amount bound, whereas in CSF the curve was sigmoidal (due to cooperativity) and shallower (due to fewer binding sites).

Specific ^{3}H-naloxone binding was greater in CSF than Tris buffer (Figure 11). In order to obtain reproducible results, binding in CSF was measured at lower concentrations of nonlabelled naloxone. The concentration of binding sites was increased in CSF (Table 3) while the dissociation constant was unchanged. Naloxone inhibition of ^{3}H-naloxone binding was shallower in CSF; this can be attributed to the decrease of cooperativity among sites which was observed. The results of saturation experiments (Figure 9) are consistent with these findings, even though results of the saturation studies were complicated by great variability. In Tris buffer, the saturable binding increased quite rapidly toward the apparent B_{max} (determined from displacement experiments), whereas in CSF, with less interaction among sites, the saturable binding was more hyperbolic.

Thus, binding of opiate agonists and antagonists is different in Tris buffer and artificial CSF. This effect is similar to the reported differential effect of sodium ion on opiate binding in brain tissue homogenates.

TABLE 3

Binding Parameters for Etorphine and Naloxone Binding to Brain Slices

	B_{max} fmoles / mg protein	K_D (nM)	n	C fmoles / mg protein
Etorphine				
Tris	119	1.72	2.0	17
CSF	68	1.68	4.2	24
Naloxone				
Tris	132	3.80	3.6	14
CSF	173	3.51	1.6	13

Binding of ^{3}H-etorphine or naloxone to brain slice preparation was determined using the displacement method. Binding parameters were determined using nonlinear least squares regression analysis of the data according to equation 2. B_{max} is the binding site concentration, K_D (dissociation constant) the concentration at which half of the sites are bound, n is the number of interacting sites, and C is the nonsaturable binding.

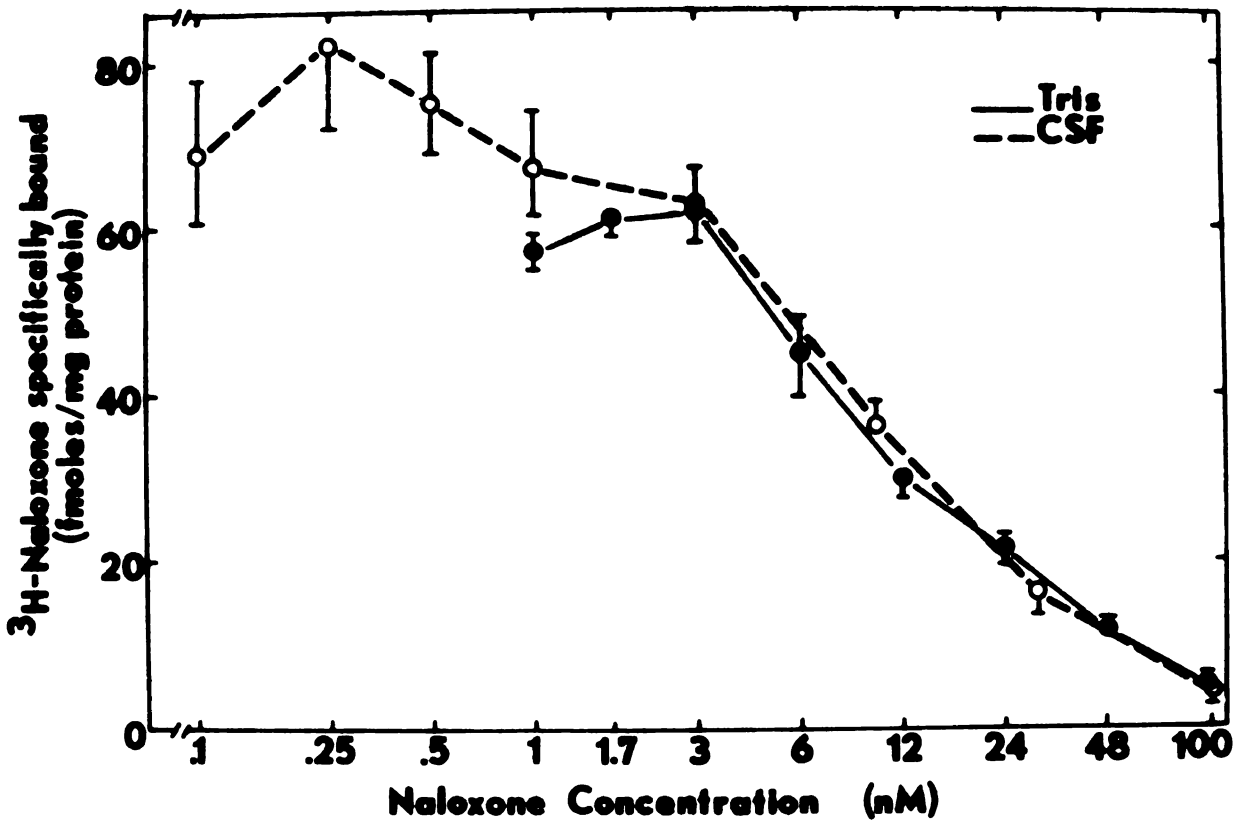


Figure 11. Binding of ^{3}H-naloxone to brain slices.

Binding of ^{3}H-naloxone (3 nM) was measured in Tris buffer and CSF, using the displacement method. The concentration of nonlabelled naloxone is shown on the abscissa. Nonsaturable binding (not shown) was determined in the presence of 10^{-5} M nonlabelled naloxone. Data are shown as means $\pm$ S.E.M. of 4 experiments. Binding constants determined for the data are shown in Table 3.

Alterations of sodium concentration and ionic strength. The differences of etorphine and naloxone binding to brain slices, observed in Tris buffer and artificial CSF, are the same as the effect of sodium ion on agonist and antagonist binding to homogenate preparations. Therefore, binding of morphine and naloxone was determined at varying sodium concentrations and in different iso-osmotic solutions.

Sodium ion inhibited agonist and enhanced antagonist binding, to brain slices, in a concentration dependent manner (Figure 12). For ^{3}H-morphine, 150 mM NaCl (in 50 mM Tris buffer), inhibited specific binding to 30% of the binding observed in Tris buffer, and intermediate inhibition was found for 50 mM (74%) and 100 mM (58%) NaCl. Specific binding of ^{3}H-naloxone was greatly enhanced by 25 mM NaCl and little increase above this was found at higher sodium concentrations. Thus, sodium ion has a differential effect, on opiate binding sites in brain slices, *in vitro*, to inhibit agonist binding and enhance antagonist binding. The differences of binding observed in artificial CSF and Tris buffer may be explained by the presence and absence of sodium in the two solutions.

In some preliminary experiments, morphine and naloxone binding was determined in several iso-osmotic buffers. Saturable ^{3}H-morphine binding in 0.32 M sucrose (Figure 13a) and in 5% (w/v) mannitol (Figure 13b) was the same as in 50 mM Tris buffer (Figure 13). Nonsaturable binding was quite high in iso-osmotic sucrose or mannitol solutions, and saturable binding accounted for less than 30% of the total binding, at the lowest concentrations of ^{3}H-morphine. In displacement experiments, iso-osmotic sucrose did not appear to enhance specific binding of ^{3}H-naloxone (Figure 14), and potassium ion (125 mM KCl in 50 mM Tris buffer) inhibited ^{3}H-naloxone binding

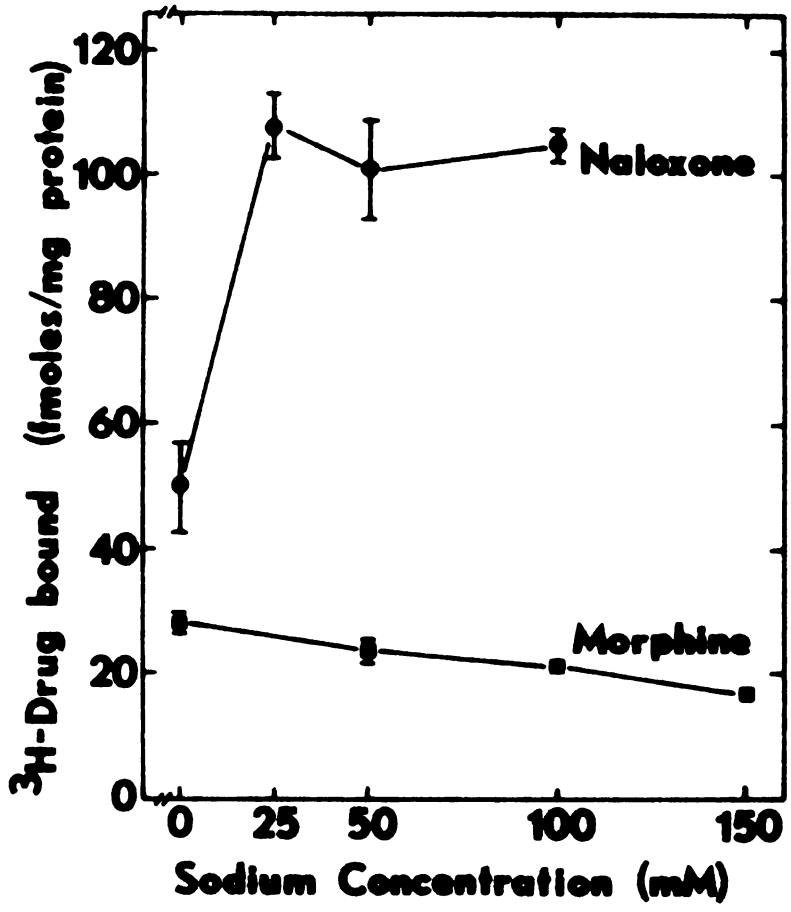


Figure 12. Effect of sodium ion on binding of ^{3}H-morphine and ^{3}H-naloxone to brain slices.

Slices were incubated in 50 mM Tris-HCl buffer with the indicated sodium concentration. Concentration of both tritiated drugs was 3 nM. Values are shown as mean ± S.E.M. of 5 (morphine) or 4 (naloxone) experiments.

Figure 13. ^{3}H-Morphine binding in iso-osmotic solutions.

Saturable binding of ^{3}H-morphine was determined as the difference between total and nonsaturable (i.e., in the presence of 10^{-5} M nonlabelled morphine) binding of ^{3}H-morphine. In Panel A results with Tris buffer are shown for comparison. In iso-osmotic sucrose (Panel A) total and nonsaturable binding were greatly increased, particularly at high ^{3}H-morphine concentrations, and saturable morphine binding could not be detected. The increase of nonsaturable binding was not as great in iso-osmotic mannitol (Panel B). In contrast, nonsaturable binding of ^{3}H-morphine was less in iso-osmotic ammonium chloride-Tris solution (Panel C) and saturable morphine binding was similar to that in Tris buffer.

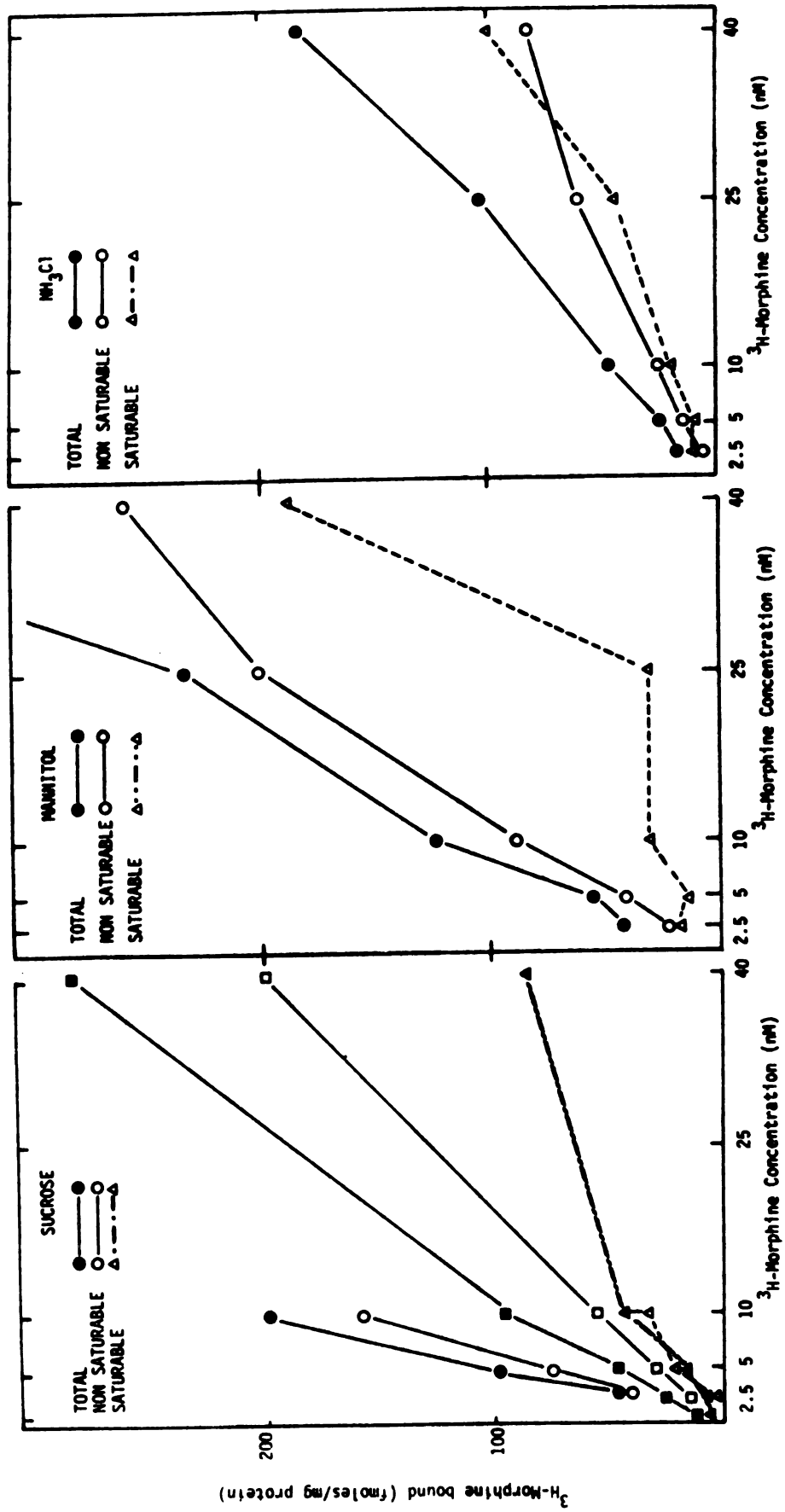

SUCROSE
TOTAL
NON SATURABLE
SATURABLE
MANNITOL
TOTAL
NON SATURABLE
SATURABLE
NH4Cl
TOTAL
NON SATURABLE
SATURABLE
3H-Morphine bound (fmoles/mg protein)
200
100
2.5 5 10 25 40
3H-Morphine Concentration (nM)

Figure 13

(Figure 14). None of these buffer solutions offers an advantage over Tris buffer; therefore, Tris buffer was used for subsequent experiments with low sodium concentration. Furthermore, it may be concluded that the differential effect of sodium on opiate binding sites, *in vitro*, is not an osmotic effect.

Interactions between etorphine and naloxone. To further characterize the influence of sodium ion on the opiate binding site, the concentration of nonlabelled naloxone necessary to inhibit specific ^{3}H-etorphine (1 nM) binding by 50 percent (IC50) was to be compared in Tris buffer and artificial CSF. However, positive cooperativity of ^{3}H-etorphine binding was apparent, in both buffers, when nonlabelled naloxone was used in the displacement experiment (Figure 15). Because specific binding of ^{3}H-etorphine was greater at 1 nM naloxone, than in the absence of naloxone, it is not valid to determine a naloxone IC50. At relatively low concentrations of nonlabelled naloxone (less than 1.7 nM), specific ^{3}H-etorphine binding was enhanced by naloxone, whereas in the presence of higher naloxone concentrations (above 3 nM) specific ^{3}H-etorphine binding was inhibited. Specific binding of ^{3}H-etorphine was less in CSF than Tris buffer and, at higher naloxone concentrations (above 6 nM), specific ^{3}H-etorphine binding indicative of cooperativity appeared more pronounced in CSF than Tris buffer. Binding constants cannot be determined from the interaction experiments, because two sets of binding constants plus inhibition factors are involved. The data do indicate that naloxone can displace ^{3}H-etorphine from some of its binding sites. However, if naloxone had access to all ^{3}H-etorphine binding sites, in both solutions, the two curves would meet at high naloxone concentrations.

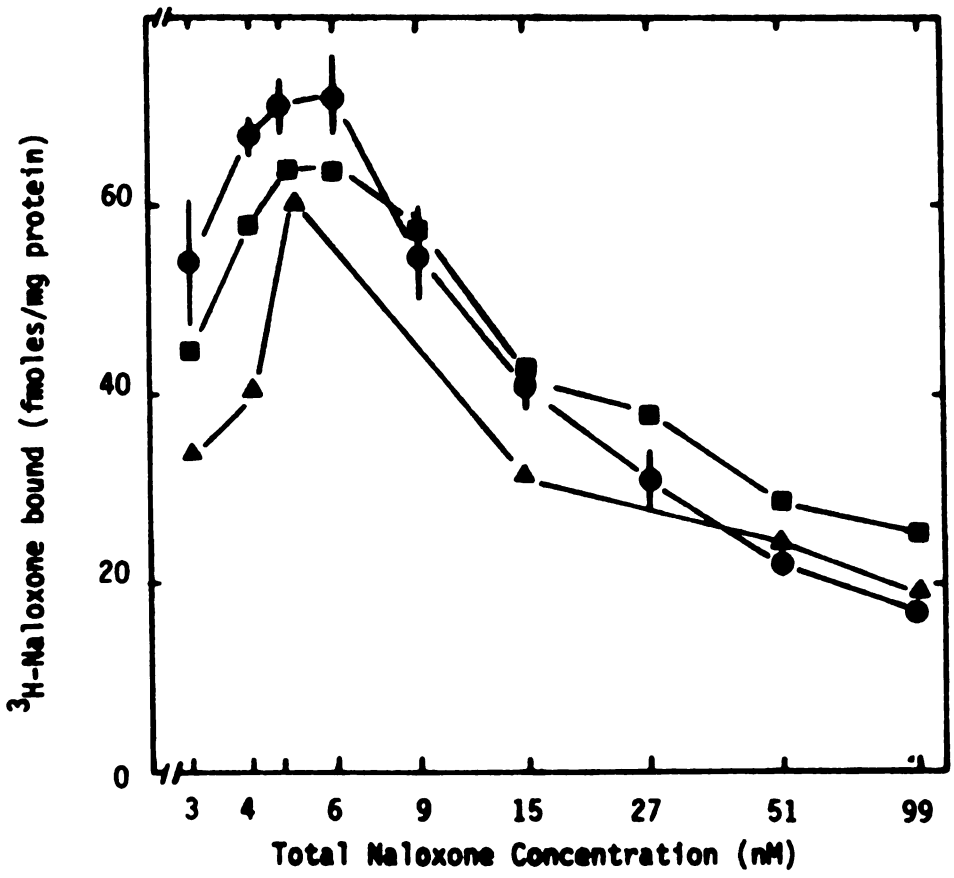


Figure 14. Effect of sucrose and potassium chloride on ^{3}H-naloxone binding to brain slices.

The displacement method was used to study binding of ^{3}H-naloxone (3 nM) in Tris buffer (●—●), 0.32 M sucrose in Tris buffer (■—■) and 125 mM KCl in tris buffer (▲—▲). Neither sucrose nor potassium mimicked the enhancement of binding observed with sodium ion.

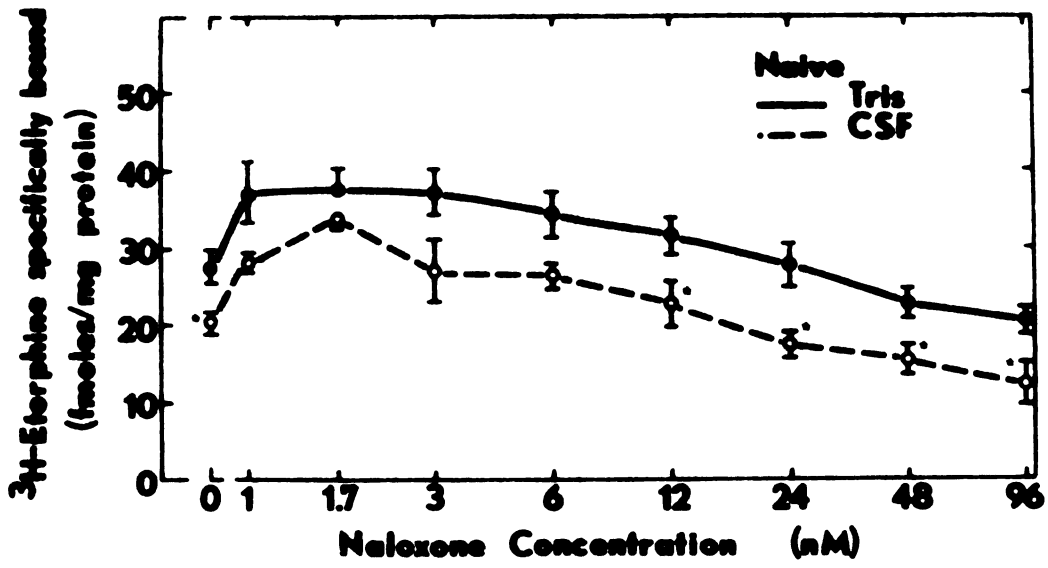


Figure 15. Interaction between naloxone and etorphine in Tris buffer and artificial CSF.

Binding of ^{3}H-etorphine (1 nM) was determined at the concentrations of unlabelled naloxone indicated. Values are shown as mean $\pm$ S.E.M. of 5 experiments.

Specific ^{3}H-etorphine binding was significantly less in CSF, above 12 nM naloxone, and closely parallel to the binding curve for Tris buffer. Thus, naloxone did not have access to a portion of the specific ^{3}H-etorphine binding sites in Tris buffer. Furthermore, naloxone induced a cooperative change in the binding site such that binding of etorphine was enhanced.

Effects of Chronic Morphine Treatment on Analgesic Receptors *in vivo* and Binding Sites *in vitro*

Analgesic tolerance. Tolerance to the analgesic effects of morphine was quantified, to determine the effectiveness of the 72 hour morphine pellet treatment regimen used, and to follow the time course of disappearance of tolerance during morphine withdrawal. Results of such studies make it possible to compare the degree of analgesic tolerance and alterations of *in vitro* binding. The analgesic effect of morphine was determined following 72 hour morphine treatment and after withdrawal from this treatment for 1, 4 and 7 days. Analgesia was assessed by warm plate (Figure 16) and foot shock (Figure 17) methods, and the data analyzed by linear regression. The slopes of the regression lines for the treatment groups were not different from control ($p<.05$). To compare the degree of tolerance between the treatment groups, the challenge dose of morphine necessary to induce a 60 second increase of reaction time in the warm plate test or a 60% change of shock threshold in the foot shock test was estimated for each group (Table 4). These points were arbitrarily chosen because they represent roughly half the maximal effect detected by the two procedures for assessing analgesia. The degree of tolerance observed was different for the two methods of measuring analgesia but,

Figure 16. Assessment of analgesic tolerance using the warm plate method.

Effect of morphine treatment and withdrawal on morphine induced analgesia measured using warm plate method. Treatments are ●—●, sham-operated controls; ○—○, 72 hour morphine pellet implantation plus 6 hours withdrawal; ▲—▲, 30 hour withdrawal; ▲—▲, 4 day withdrawal; □—□, 7 day withdrawal. Data were analyzed by linear least squares regression. The lines for treatment groups are parallel with the sham-operated control group. The estimated doses to increase the reaction time by 60 seconds are given in Table 4.

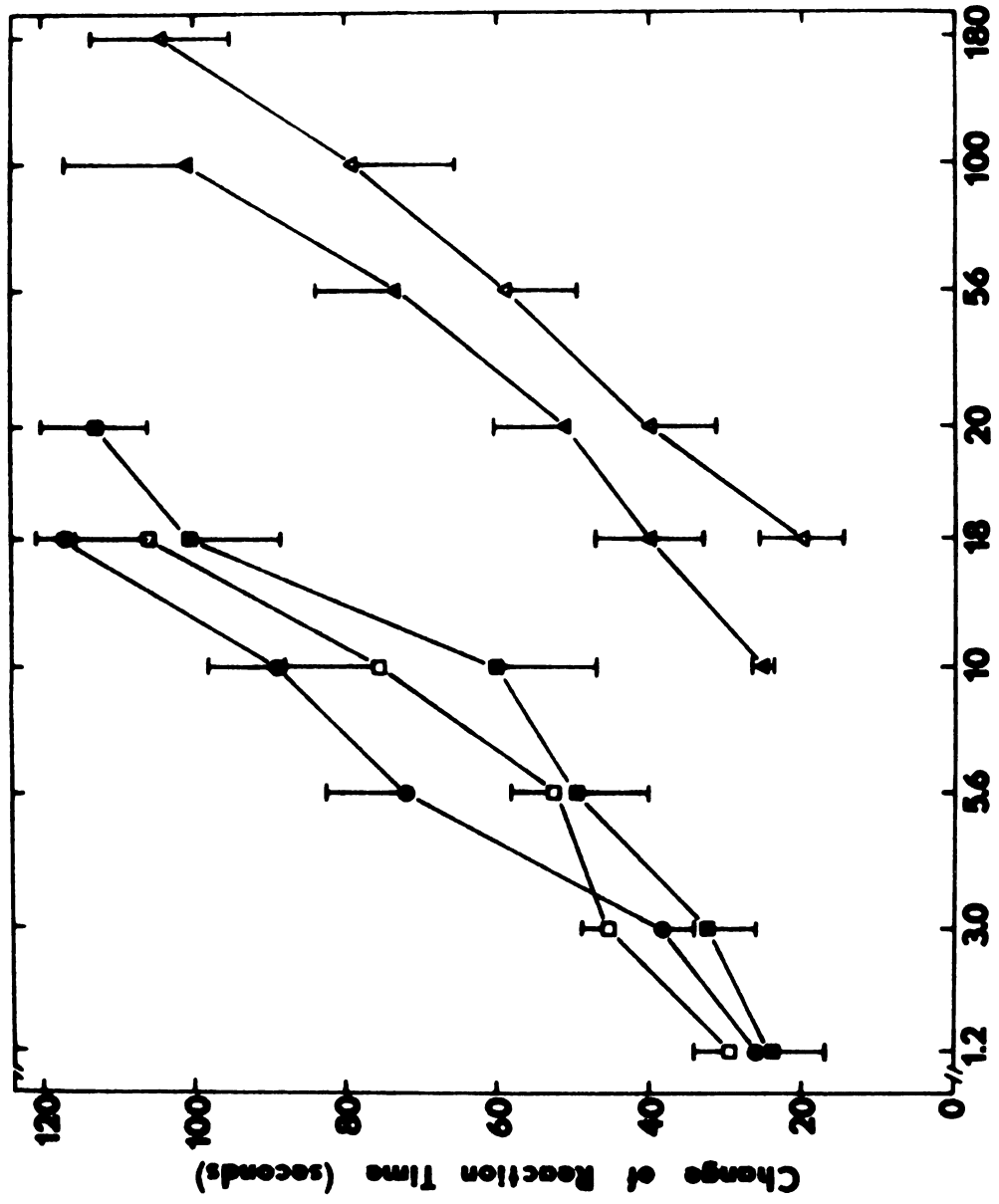

Change of Reaction Time (seconds)
120
100
80
60
40
20
0
1.2
3.0
5.6
10
18
20
56
100
180
Test Dose of Morphine (mg/kg)

Figure 16

Figure 17. Assessment of analgesic tolerance using the foot shock method.

Treatments were the same as Figure 16 (dfferent animals were used). The estimated doses to increase the reaction time by 60% are given in Table 4.

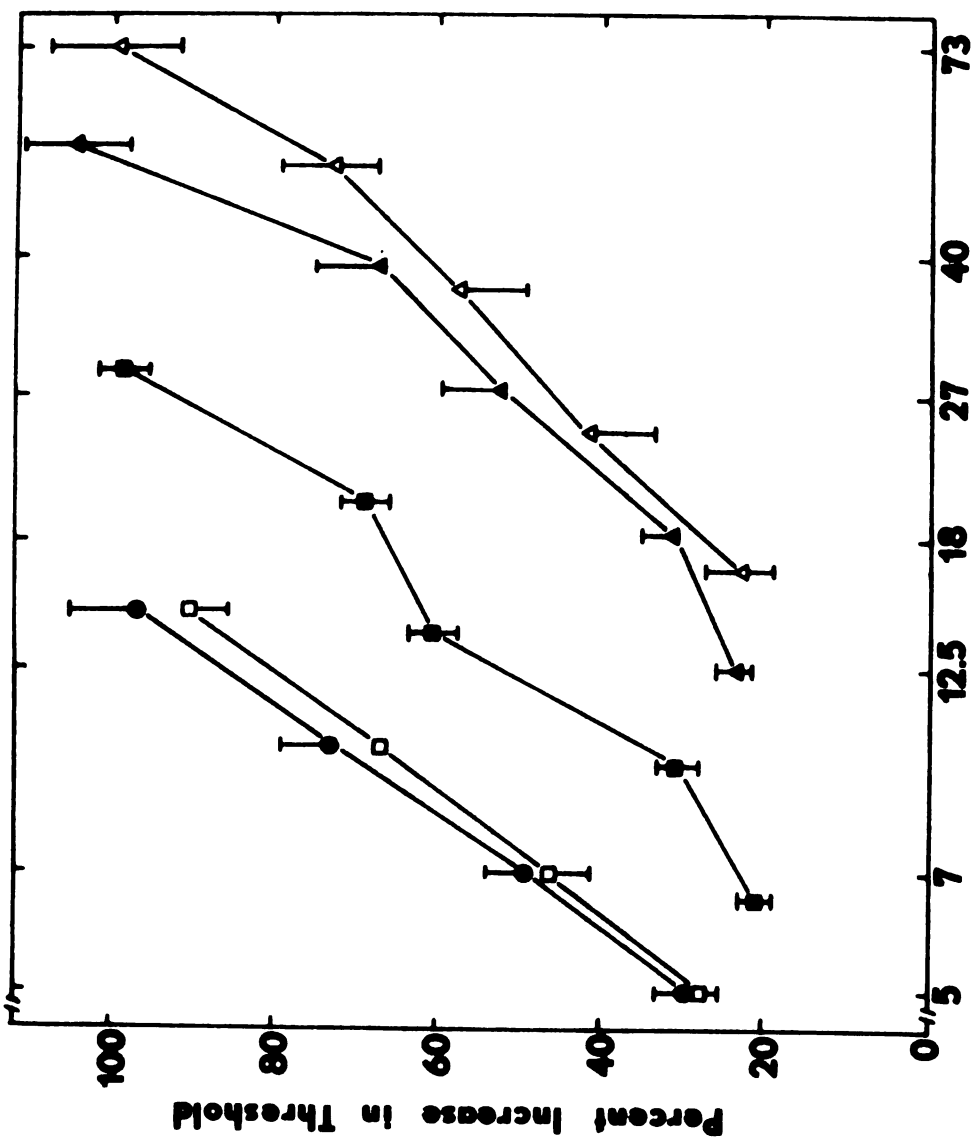

Percent Increase in Threshold
0
20
40
60
80
100
5
7
12.5
18
27
40
73
Test Dose of Morphine (mg/kg)

Figure 17

TABLE 4

Effect of Chronic Morphine Treatment and Withdrawal from Morphine on Morphine-Induced Analgesia Assessed by Warm Plate and Foot Shock Methods

	Warm Plate		Foot Shock	
Treatment	slope*	dose** (95% C.I.)	slope*	dose** (95% C.I.)
Control	90	3.5 (1- 11)	110	7.8 (2-28)
72 hr morphine	82	55.7 (14-229)	114	36.0 (18-71)
Withdrawal				
1 day	75	36.3 (6-233)	122	29.1 (16-53)
4 day	76	6.4 (1- 34)	118	15.0 (11-21)
7 day	72	4.8 (1- 20)	113	8.6 (6-12)

*Slope determined by linear regression of all the data points using an Olivetti Programma 101 microcomputer.

**Dose to increase reaction time by 60 seconds in the warm plate test or by 60% in the foot shock test was estimated from the data using linear regression techniques (see Sokal and Rohlf, 1969).

for both, the correlation between morphine treatment or duration of withdrawal and the change of estimated dose was good. For the warm plate test, 72 hour morphine treatment caused a 16-fold increase of the dose of morphine to produce a comparable degree of analgesia in control; this increase was significant ($p<.05$). During withdrawal, sensitivity to the analgesic effect of morphine was recovered. Using the foot shock method to measure analgesia, tolerance was less pronounced; following 72 hour morphine treatment, the analgesic dose was increased 5-fold. The difference in tolerance detected by the two methods is not unexpected, because both tests are based on behavioral reactions, which are also influenced by morphine.

From these studies it may be concluded that 72 hour morphine pellet treatment schedule, used in subsequent binding studies, is sufficient to induce significant tolerance to the analgesic effect of morphine. Furthermore, chronic morphine treatment induced a parallel shift of the log dose-response curve such that the morphinized rats were less sensitive to the analgesic effects of morphine. This observation is consistent with the hypothesis that narcotic tolerance is mediated by a reduction of the receptor affinity for opiate analgesics.

Morphine binding during the development and loss of tolerance. A receptor-mediated mechanism for tolerance is suggested by analgesia studies, such as those discussed above, and by electrophysiological studies of the effects of directly applied narcotic agonists and antagonists. Since binding is different if the tissue is not homogenized prior to the binding reaction, the effects of chronic morphine treatment on opiate binding, to brain slices, were determined. In

the initial saturation experiments, the homogenate preparation was also used. Saturable morphine binding was significantly decreased in brain slices prepared from rats treated for 72 hours with morphine by pellet implantation (Figure 18). This reduction was not observed in the homogenate preparation (Figure 19). The nonsaturable binding was not affected by the morphine treatment. Analysis by Scatchard plot (Table 5) indicated that binding affinity was reduced 4-fold (K_D increased in tolerant rats), while the binding site concentration was relatively unaffected by chronic morphine treatment. These results, in the initial slice experiments, were also observed in saturation experiments with the modified experimental protocol (Figure 20). As already noted, the opiate binding sites, in the slice preparation, are not independent of each other, but rather exhibit positive cooperativity. Therefore, the effect of chronic morphine treatment on morphine binding, to brain slices, was assessed using the displacement method.

Furthermore, if the reductions of morphine binding observed are related to tolerance, then the reduction of binding should correlate with the degree of tolerance. Changes of ^{3}H-morphine binding over the development and offset of tolerance were followed, using the displacement method and tissue slices (Figures 21 and 22). Binding was reduced by 36 hour morphine treatment (1 pellet), and even more so by 72 hour treatment (2 pellets). During withdrawal, binding returned gradually toward control values. Thus, for the 72 hour morphine group, ^{3}H-morphine binding was reduced, significantly, at the 3, 4, 4.7 and 6 nM concentrations, whereas after 4 days of withdrawal only the decrease at the 3 nM point was significant (Figure 22). The nonsaturable binding measured was not affected by morphine

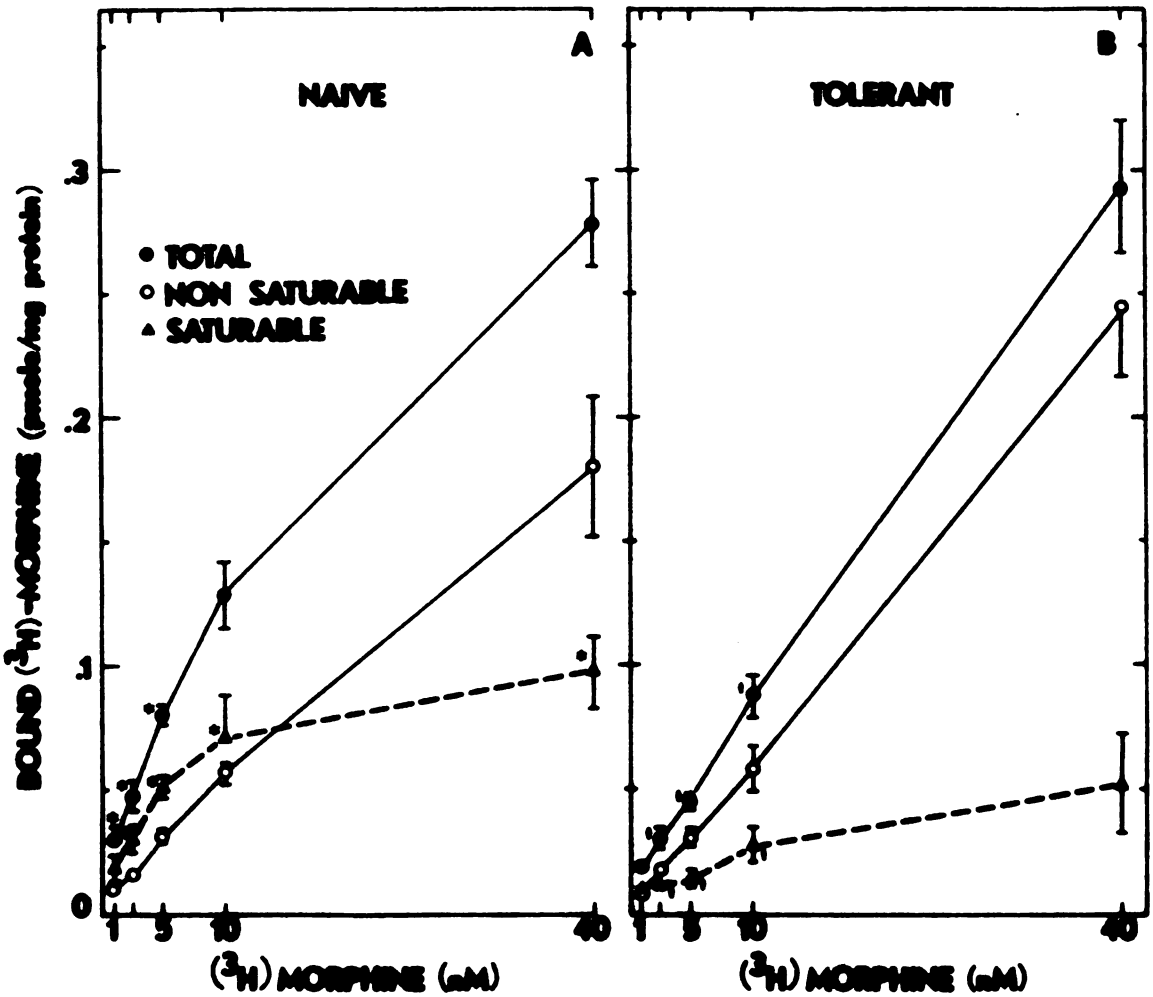


Figure 18. Initial studies on the effect of chronic morphine treatment on morphine binding: slice preparation.

Saturation method was used. Nonsaturable binding of ^{3}H-morphine was determined in the presence of nonlabelled morphine (10^{-5} M). Saturable binding was calculated as the difference between total and nonsaturable binding of ^{3}H-morphine. Animals were treated with morphine by 72 hour morphine pellet implantation. Values are shown as mean $\pm$ S.E.M. of at least 4 experiments. † indicates different from respective value in naive ($p<.05$). Binding constants are given in Table 5.

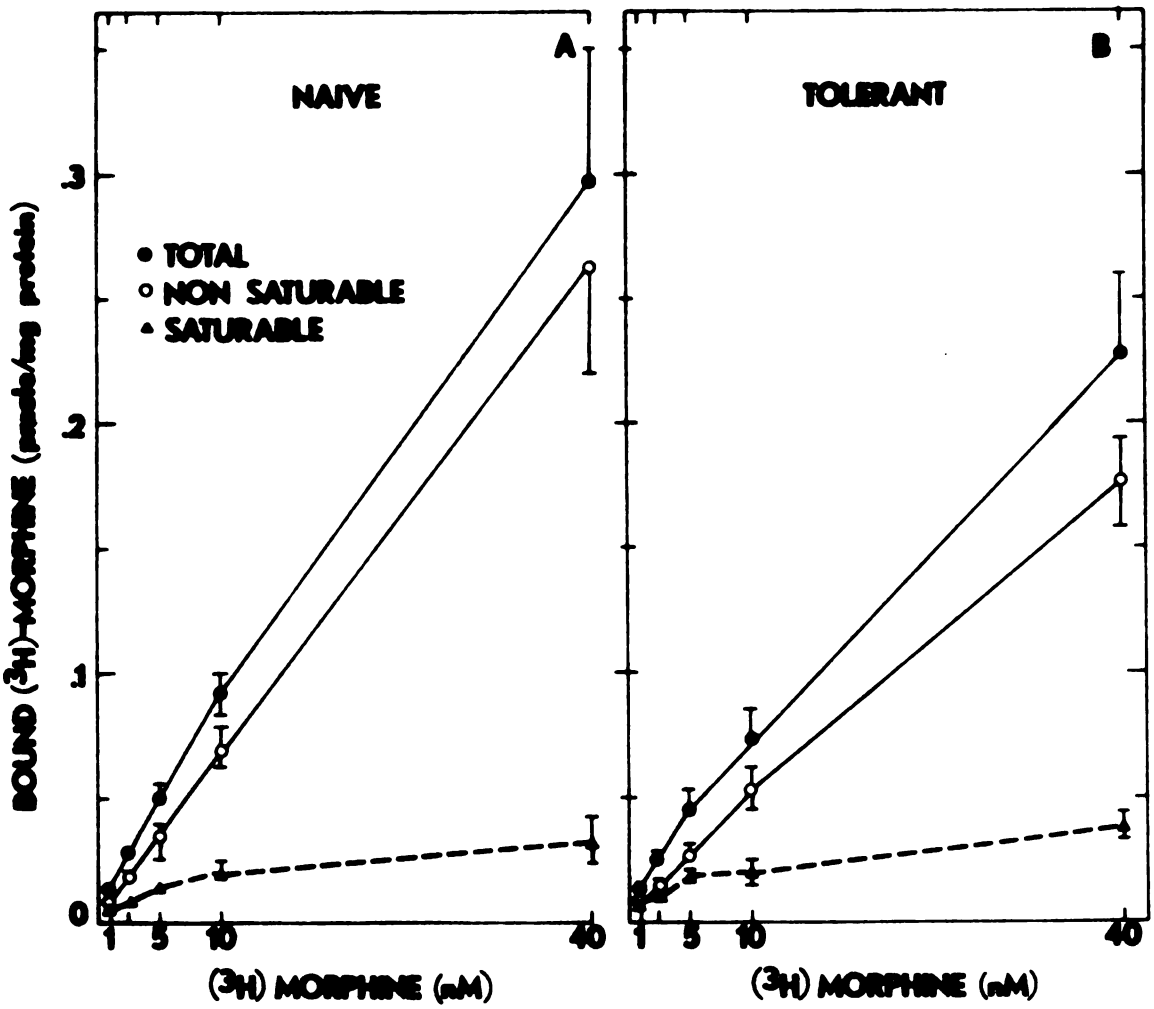


Figure 19. Initial studies on the effect of chronic morphine treatment on morphine binding: homogenate preparation.

The experimental design was the same as for Figure 18, except that the homogenate preparation was used. No significant differences between naive and tolerant groups were found.

TABLE 5

Effect of Chronic Morphine Treatment on
Morphine Binding Constants*

		K_D (nM)	B_{max} fmoles / mg protein
Homogenate	naive	9.7	42
	tolerant	9.1	46
Slice-initial	naive	6.6	120
	tolerant	22.6	80
Slice-modified	naive	19.4	105
	tolerant	48.0	145

*Estimated from Scatchard plots. The lines of best fit were determined by linear regression. The saturable binding data shown in Figures 18 (slice-initial), 19 (homogenate) and 20 (slice-modified) were used.

Figure 20. Effect of chronic morphine treatment on morphine binding: modified slice protocol.

The experiment shown in Figure 18 was repeated using the modified slice protocol. * denotes different from respective value in naive ($p<.05$). Binding constants are given in Table 5.

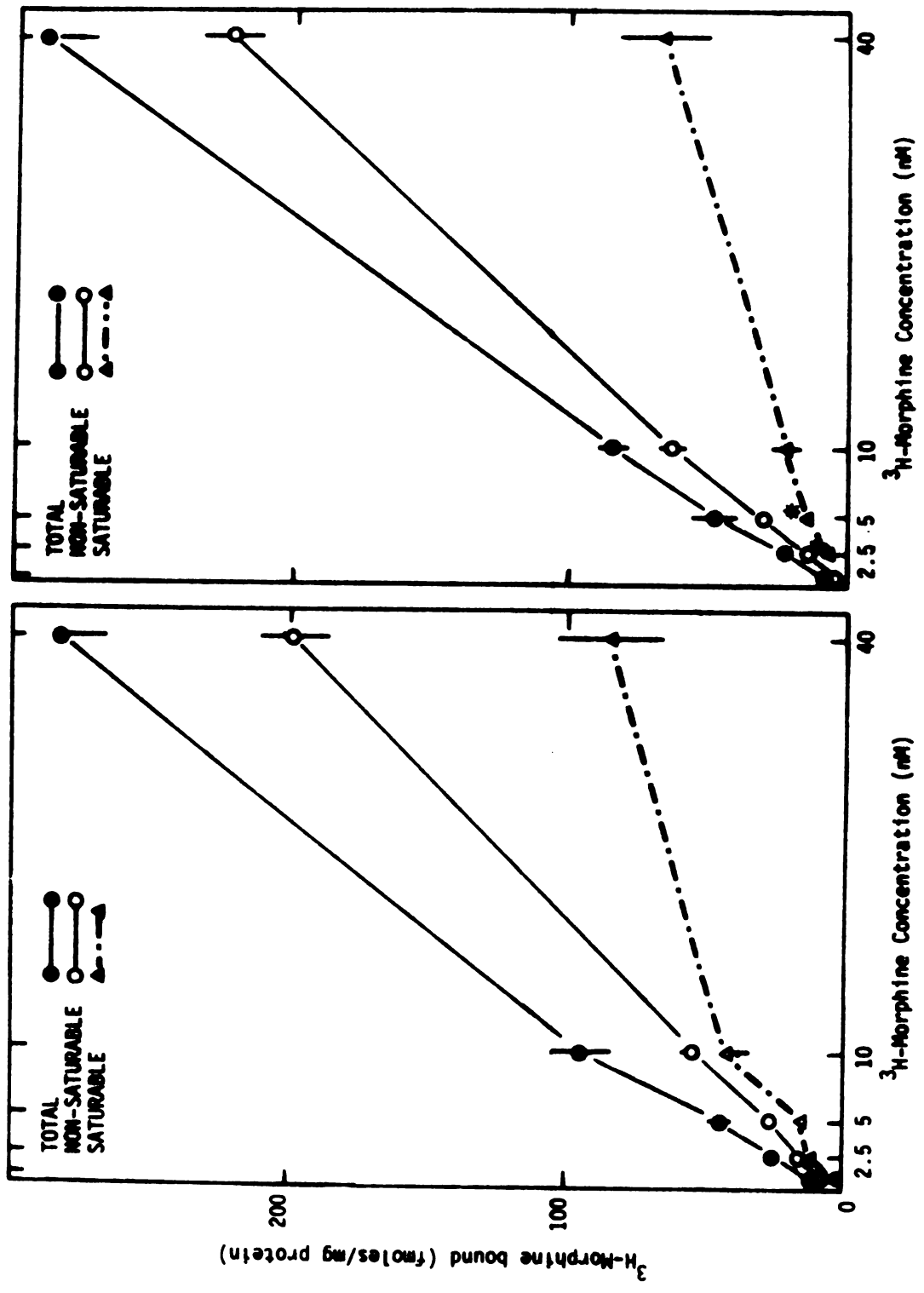

TOTAL
NON-SATURABLE
SATURABLE
^{3}H-Morphine bound (fmoles/mg protein)
0
100
200
2.5
5
10
40
^{3}H-Morphine Concentration (nM)

Figure 20

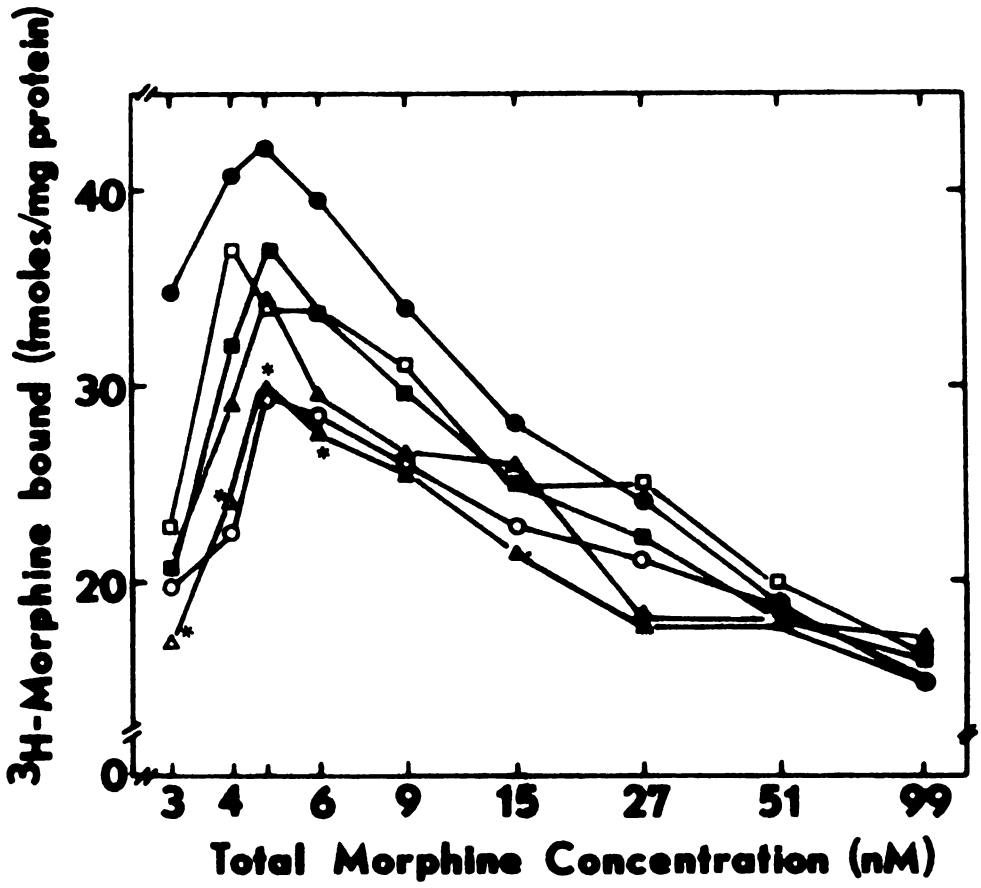


Figure 21. Morphine binding during induction of tolerance and after withdrawal from morphine.

^{3}H-Morphine (3 nM) binding was determined in displacement experiments. The total concentration of morphine in each incubation mixture is given on the abscissa. Values are shown as mean of experiments; in Figure 22 mean ± S.E.M. are shown. Treatments are ●, naive; o, 36 hour and Δ, 72 hour morphine pellet treatment; ▲, 1 day, Δ, 4 day and □, 7 day withdrawal after 72 hour morphine treatment. Binding constants for the data are given in Table 6. * indicates significant difference ($p<.05$) between naive and 72 hour morphine treatment groups.

Figure 22. Morphine binding during induction of tolerance and after withdrawal from morphine. Same data as shown in Figure 21. * indicates significant difference between treatment group and naive.

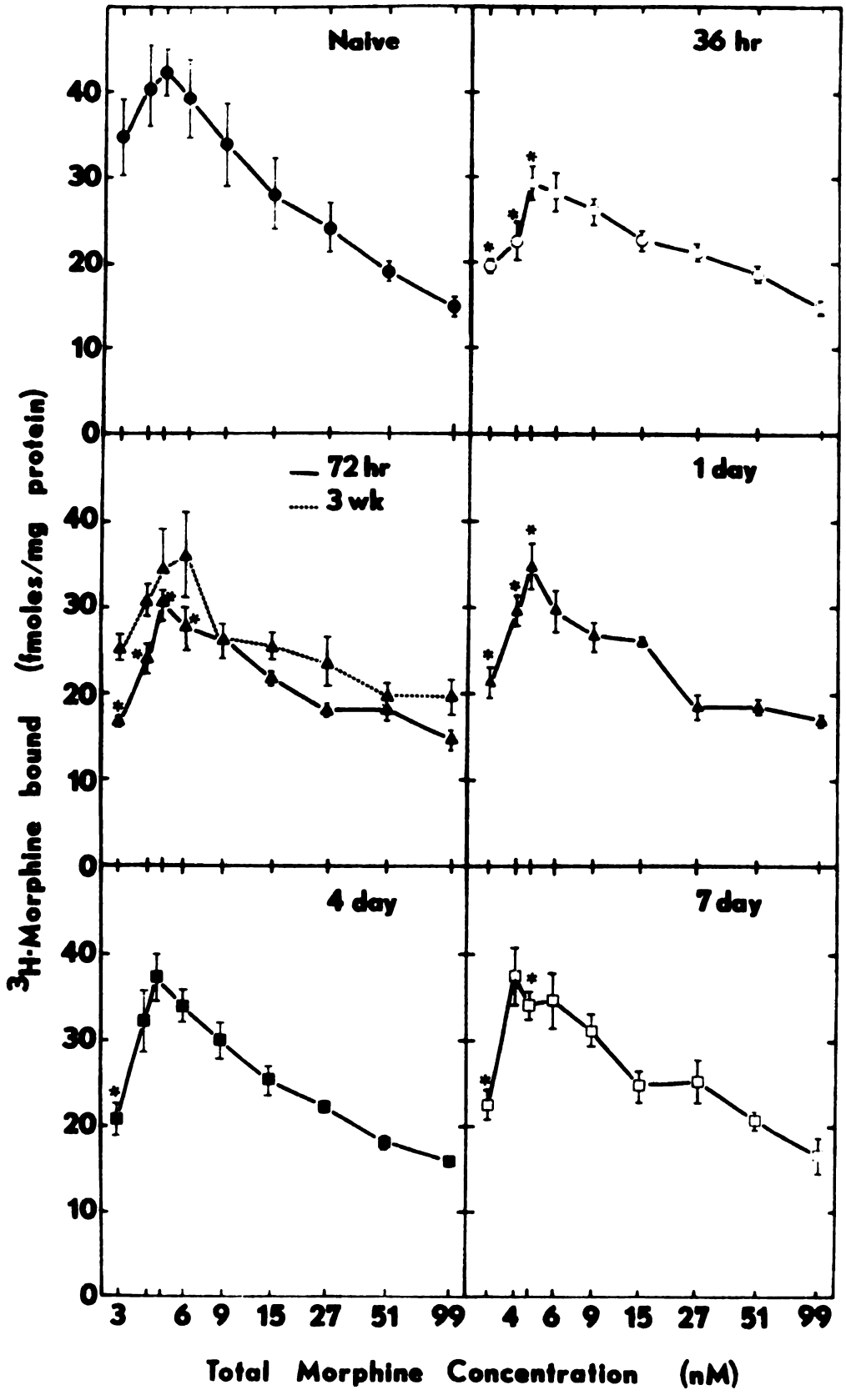

Naive
36 hr
72 hr
3 wk
1 day
4 day
7 day
^{3}H-Morphine bound (fmoles/mg protein)
0
10
20
30
40
3
6
9
15
27
51
99
4
6
9
15
27
51
99
Total Morphine Concentration (nM)

Figure 22

treatment. The initial increase of ^{3}H-morphine binding, in the presence of low concentrations of nonlabelled morphine, indicative of cooperativity, was found for all treatment groups, and appeared to be reversibly enhanced in the tolerant state (Table 6, n_{app} increased following morphine treatment and returned toward control values during withdrawal). The seven day withdrawal group did not fit this pattern. The nonsaturable binding was greater and calculated cooperativity was more pronounced, apparently because binding of ^{3}H-morphine, in the absence of nonlabelled morphine, was quite low relative to binding in the presence of nonlabelled morphine. The reasons for this are not clear. The dissociation constant was not markedly or consistently affected by chronic morphine treatment; the binding site concentration was reduced (Table 6). Because of the variability inherent in using slices from different brain regions, all of the data points for a given treatment were analyzed as a group. Thus, only one set of binding constants was determined for each treatment. The variability around the constants is not known. Therefore, it is not possible to make statistical comparisons of the binding constants.

Although morphine treatment by 72 hour pellet implantation is sufficient to induce a significant degree of tolerance, the duration of treatment is short. Therefore, one group of animals was treated with morphine by subcutaneous injection for 3 weeks. Morphine was given either continuously or 96 times per day, to prevent daily withdrawal (which is frequently observed if morphine is given only 2 or 3 times a day). ^{3}H-Morphine binding was measured by the displacement method and the results (Figure 22 and Table 6) were similar to those obtained with 72 hour pellet treatment. Binding was reduced and

TABLE 6

Effect of Chronic Morphine Treatment on Binding Parameters for Morphine Binding to Brain Slices

	B_{max} $\frac{\text{fmoles}}{\text{mg protein}}$	K_D (nM)	n_{app}	C $\frac{\text{fmoles}}{\text{mg protein}}$
Naive	64	3.8	3.1	15
36 hr Morphine	31	4.5	5.8	16
72 hr Morphine	28	4.1	11.0	15
1 d Withdrawal	30	3.8	8.0	17
4 d Withdrawal	39	3.9	7.7	16
7 d Withdrawal	29	3.5	12.3	19
3 wk Morphine	32	3.9	5.9	19

Binding of ^{3}H-morphine to brain slice preparations was determined using the displacement method. Binding parameters were estimated using nonlinear least squares regression analysis of the data according to equation 2. B_{max} is the binding site concentration, K_D (dissociation constant) the concentration at which half of the sites are bound, n is the number of interacting sites, and C is the nonsaturable binding. Rats received one (36 hr group) or two morphine pellets implanted subcutaneously or subcutaneous injection of morphine for 3 weeks via an indwelling catheter. Withdrawal was initiated by removing the pellets after 72 hr morphine treatment.

cooperativity was still apparent, and somewhat increased. Thus, the 72 hour pellet implantation induced the same changes of binding as did the more traditional chronic injection treatment. Furthermore, reductions of agonist binding observed after short term morphine treatment apparently can be maintained if exposure to drug was continued. However, the alterations of agonist binding were reversible after the morphine treatment was terminated.

In the slice saturation experiments, binding of ^{3}H-morphine was reduced in chronically morphine-treated animals and this was attributed to an increase of the K_D, or decreased binding affinity. When compared to control, saturable ^{3}H-morphine binding was more sigmoidal after 72 hour morphine treatment (and this holds for the initial and modified binding protocol experiments, Figures 18 and 20). Thus, with two different types of binding experiments, chronic morphine treatment, which results in developments of tolerance to morphine, was associated with a reduction of saturable or specific binding of ^{3}H-morphine to brain slices.

Effect of sodium on *in vitro* binding in tolerant animals. The reduction of morphine binding observed following chronic morphine treatment is similar to the reduction of agonist binding observed in physiological concentration of sodium ion. Therefore, the effect of sodium ion on the binding site, in brain slices, was determined following morphine treatment.

In the etorphine displacement experiment, chronic morphine treatment reduced the amount of ^{3}H-etorphine binding observed in either Tris buffer (Figure 23) or artificial CSF (Figure 24). This effect was more pronounced, and reached the criterion for statistical

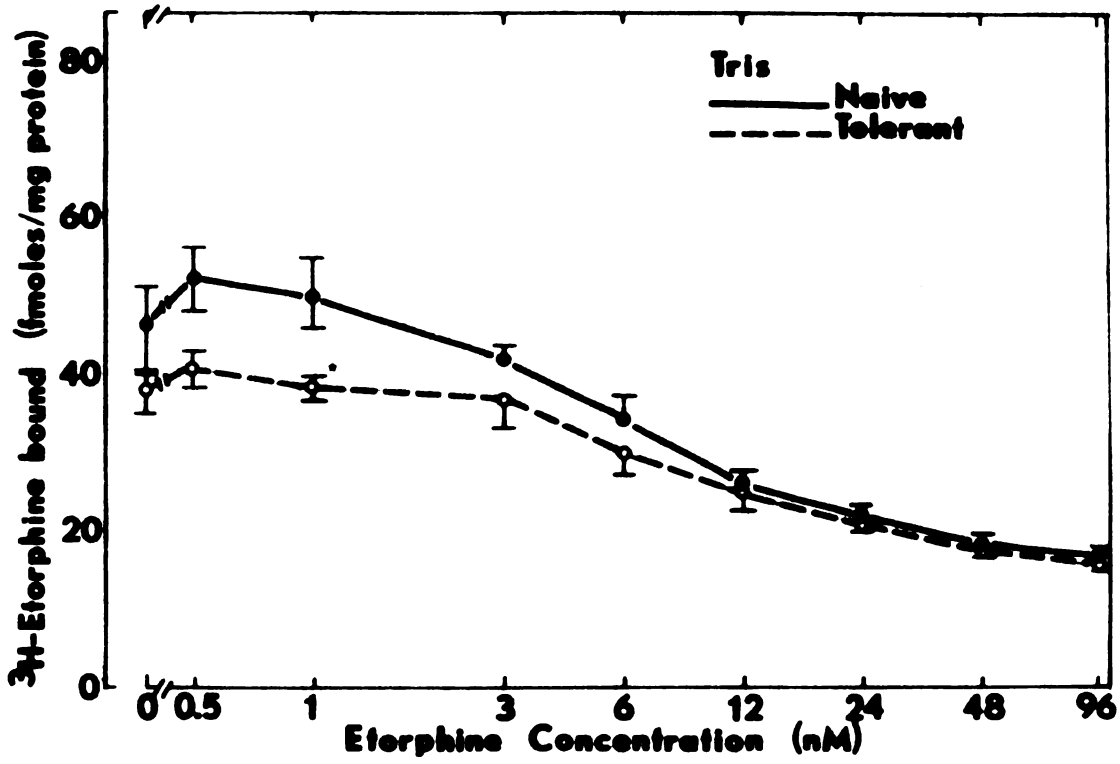


Figure 23. Effect of chronic morphine treatment on etorphine binding in Tris buffer.

^{3}H-Etorphine (1 nM) binding was determined in displacement experiments with Tris buffer as the incubation medium. The concentration of nonlabelled etorphine is shown on the abscissa. Rats were made tolerant by 72 hour pellet implantation. Values are shown as mean $\pm$ S.E.M. of 5 (naive) and 4 (tolerant) experiments. * indicates different from respective value in naive ($p<.05$); the difference at 0.5 nM etorphine just missed statistical significance. Binding constants for the data are given in Table 7.

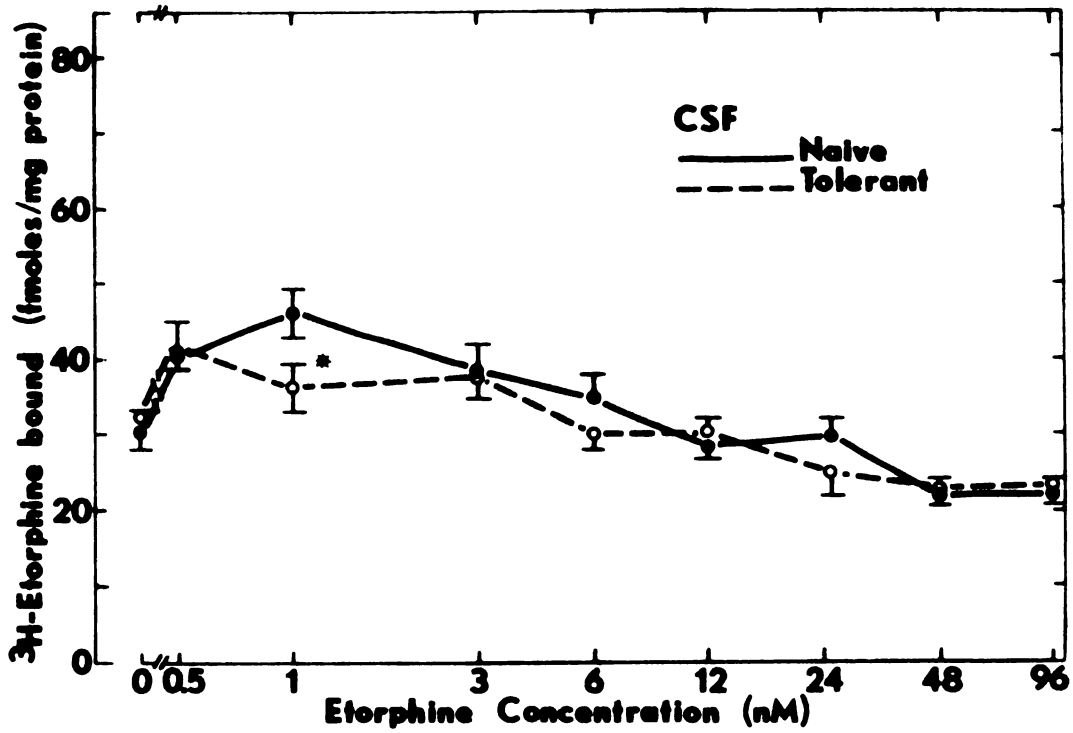


Figure 24. Effect of chronic morphine treatment on etorphine binding in artificial CSF.

The experiment was the same as in Figure 23, except that CSF was used and was replicated 4 times for both groups.

significance, at the lower concentrations of nonlabelled etorphine. The concentration of binding sites and the nonsaturable binding, determined by nonlinear regression analysis, were unaffected by morphine treatment (Table 7). The binding affinity was decreased (dissociation constant increased) and degree of cooperativity decreased in the treated groups, in both incubation solutions. As previously discussed, in naive rats the concentration of etorphine binding sites was decreased and the degree of interaction among these sites increased, in artificial CSF. The same relationship was found, for tolerant animals, in Tris buffer and CSF. However, the reduction of etorphine binding following morphine treatment was associated with a reduction of binding affinity and cooperativity, and thus does not resemble the effect of sodium.

The effects of chronic morphine treatment on naloxone binding were different in Tris buffer and CSF. In Tris buffer, ^{3}H-naloxone binding was significantly less in tolerant animals, at the lowest concentrations of nonlabelled naloxone (Figure 25, 1-3 nM points). The concentration of binding sites was the same in naive and tolerant animals (Table 8). The initial increase of ^{3}H-naloxone binding at low concentrations of nonlabelled naloxone, although apparent, was blunted in the tolerant animals, and this is reflected in the decrease of the apparent number of interacting sites (from 3.6 to 2.2). The dissociation constant was greater after chronic morphine treatment, indicating that binding affinity for naloxone was reduced following 72 hour exposure to morphine. A similar effect was observed in saturation experiments (Figure 26). The saturable binding was significantly reduced in the morphine treated group, and the curve was shallower and more hyperbolic. This supports the observations from the displacement

TABLE 7

Effect of Chronic Morphine Treatment on Binding Parameters for Etorphine Binding to Brain Slices

		B_{max} fmoles / mg protein	K_D (nM)	n_{app}	C fmoles / mg protein
Tris	naive	119	1.7	2.0	17
	tolerant	140	3.1	1.5	16
CSF	naive	68	1.7	4.2	24
	tolerant	68	2.0	2.3	23

TABLE 8

Effect of Chronic Morphine Treatment on Binding Parameters for Naloxone Binding to Brain Slices

		B_{max} fmoles / mg protein	K_D (nM)	n_{app}	C fmoles / mg protein
Tris	naive	132	3.8	3.6	14
	tolerant	141	5.7	2.2	13
CSF	naive	173	3.5	1.6	20
	tolerant	115	3.1	5.1	28

Binding of ^{3}H-etorphine or naloxone to brain slice preparations was determined using the displacement method. Binding parameters were estimated using nonlinear least squares regression of the data according to equation 2. B_{max} is the binding site concentration, K_D (dissociation constant) is the concentration at which half of the sites are bound, n is the number of interacting sites, and C is the nonsaturable binding. Rats were treated by 72 hour pellet implantation.

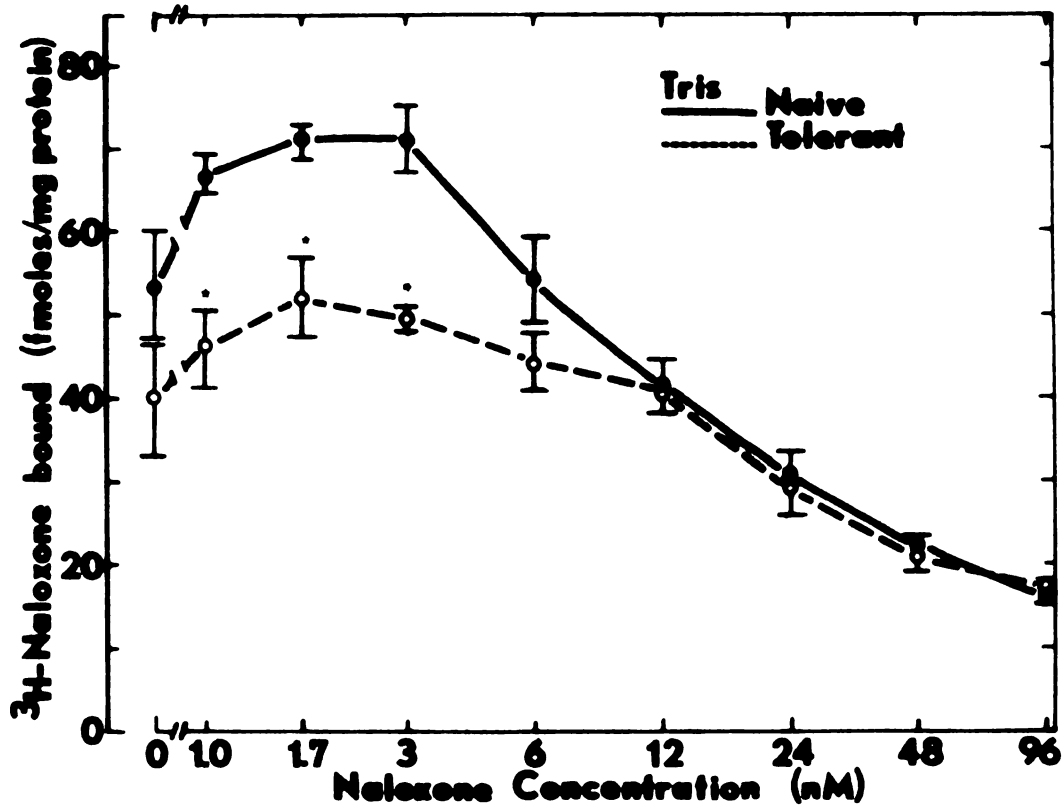


Figure 25. Effect of chronic morphine treatment on naloxone binding in Tris buffer.

^{3}H-Naloxone (3 nM) binding was determined in displacement experiments with Tris buffer as the incubation medium. The concentration of nonlabelled naloxone is shown on the abscissa. Rats were treated (for the tolerant group) by 72 hour morphine pellet implantation. Values are shown as mean $\pm$ S.E.M. of 4 (naive) and 5 (tolerant) experiments. * indicates different from respective value in naive ($p<.05$). Binding constants for the data are given in Table 8.

Figure 26. The effect of chronic morphine treatment on saturable naloxone binding in Tris buffer.

Saturable ^{3}H-naloxone binding was calculated as the difference between binding of ^{3}H-naloxone observed in the presence (nonsaturable) and absence of 10^{-5} M nonlabelled naloxone. Animals were treated by 72 hour morphine pellet implantation. Values are shown as mean $\pm$ S.E.M. of 4-6 experiments. * denotes different from respective in naive animals ($p<.05$).

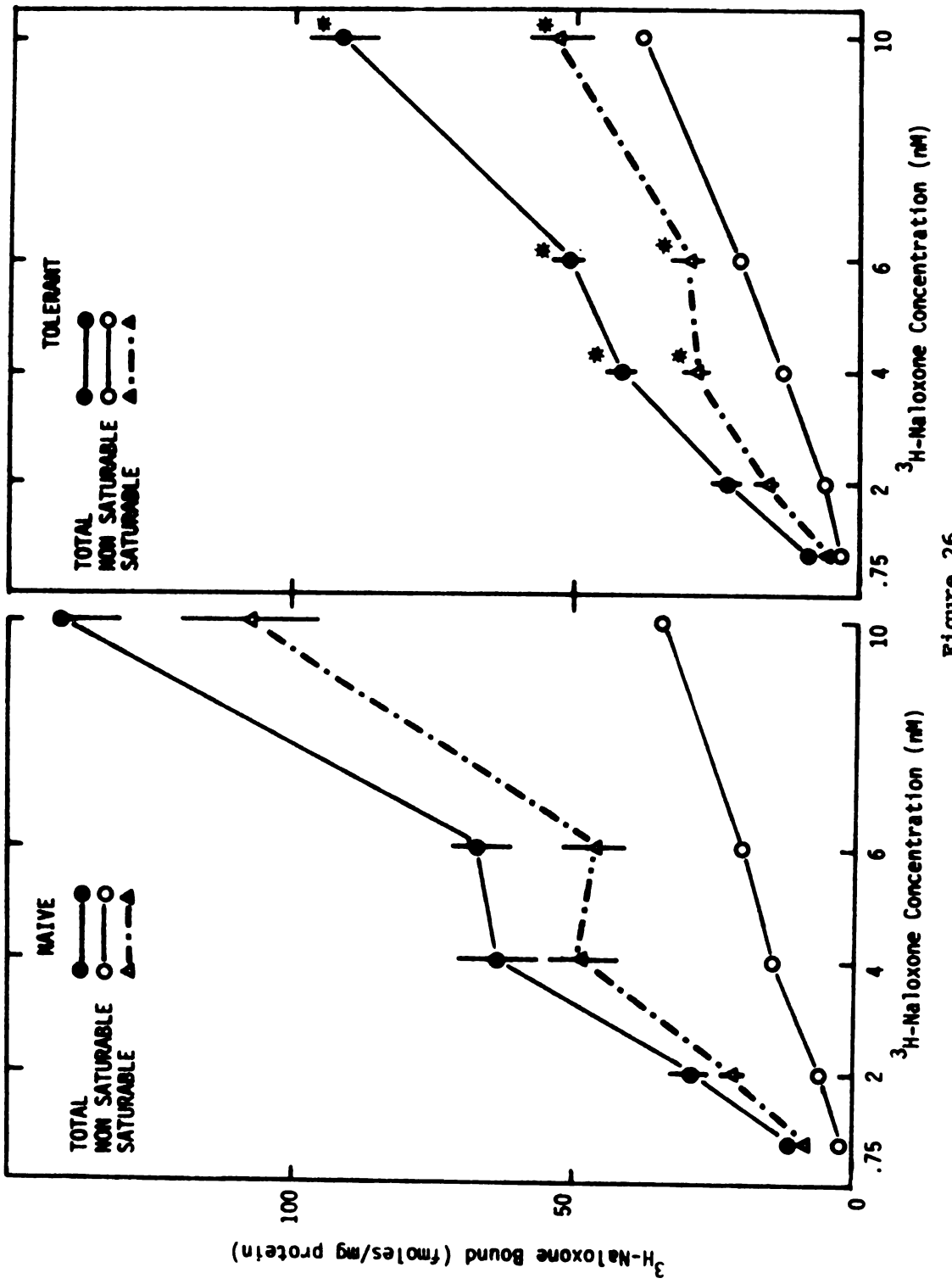

NAIVE
TOTAL
NON SATURABLE
SATURABLE
TOLERANT
TOTAL
NON SATURABLE
SATURABLE
^{3}H-Naloxone Bound (fmoles/mg protein)
0
50
100
.75
2
4
6
10
^{3}H-Naloxone Concentration (nM)

Figure 26

experiments that chronic morphine treatment results in decreased affinity of the binding sites for naloxone as well as decreased interaction among binding sites.

Chronic morphine treatment had little effect on ^{3}H-naloxone binding in CSF (Figure 27). The binding site concentration was slightly reduced and the dissociation constant was unchanged (Table 8). The apparent interaction among sites was increased after chronic morphine treatment. This may account for the more sigmoidal shape of the saturable binding curve (Figure 28) in tolerant animals. In contrast to the results for etorphine, the relationship of binding parameters in the presence and absence of sodium (i.e., in CSF and Tris buffer) was not the same for naive and tolerant rats. In naive rats, the dissociation constant was the same in CSF, the concentration of binding sites increased, and cooperativity among sites decreased. In morphine treated rats, the dissociation constant and binding site concentration were both less, and cooperativity increased, in CSF.

Stimulation of ^{3}H-naloxone binding and inhibition of ^{3}H-morphine binding by sodium was observed after chronic morphine treatment (Figure 29). Significantly less ^{3}H-naloxone binding was observed at all sodium concentrations (although in the absence of sodium this difference just missed the $p<.05$ criterion) in the treated group. Between 0 and 25 mM sodium the stimulation was quite marked, and thus results were variable. When expressed as percent of zero sodium, the enhancement by increasing sodium concentrations was greater in tolerant animals, possibly because binding was initially lower, but reached the same amount bound in high sodium. Similarly, ^{3}H-morphine binding was reduced in chronically treated animals, at all sodium

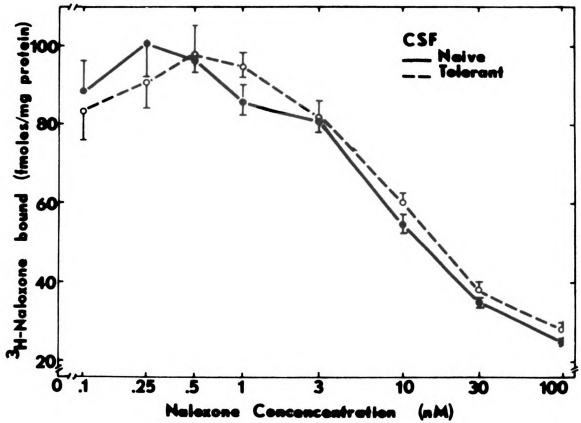


Figure 27. Effect of chronic morphine treatment on naloxone binding in artificial CSF solution.

The experiment was the same as in Figure 25, except that CSF was used and the experiment was replicated 6 times for both groups. No significant differences were found.

Figure 28. The effect of chronic morphine treatment on saturable naloxone binding in artificial CSF.

Saturable ^{3}H-naloxone binding was calculated as the difference between binding of ^{3}H-naloxone observed in the presence (nonsaturable) and absence (total) of 10^{-5} M nonlabelled naloxone. Animals were treated by 72 hour morphine pellet implantation. Values are shown as mean $\pm$ S.E.M. of 4-6 experiments. No significant difference between naive and tolerant groups was found.

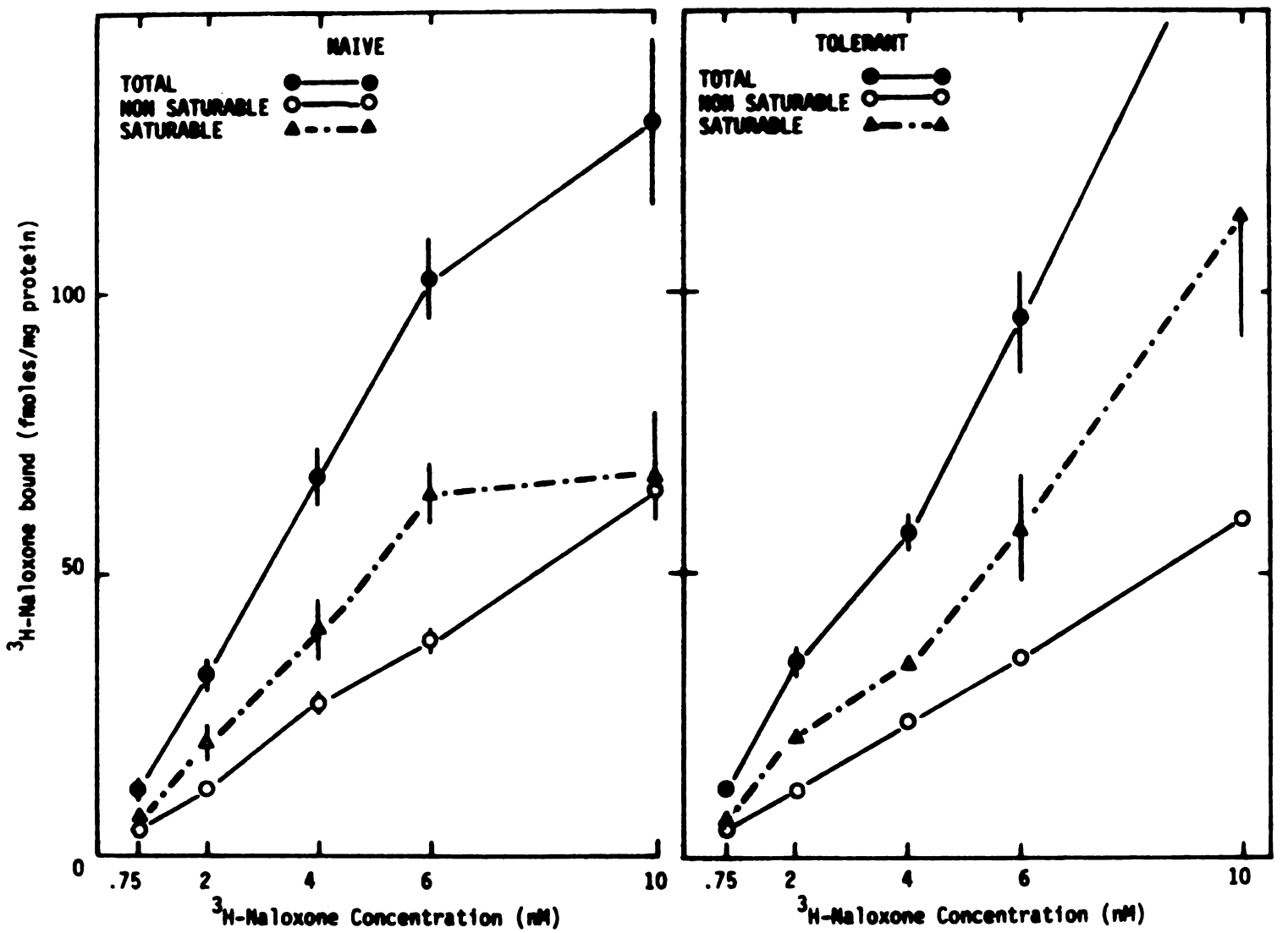

NAIVE
TOTAL
NON SATURABLE
SATURABLE
TOLERANT
TOTAL
NON SATURABLE
SATURABLE
^{3}H-Naloxone bound (fmoles/mg protein)
0
50
100
.75
2
4
6
10
^{3}H-Naloxone Concentration (nM)
^{3}H-Naloxone Concentration (nM)

Figure 28

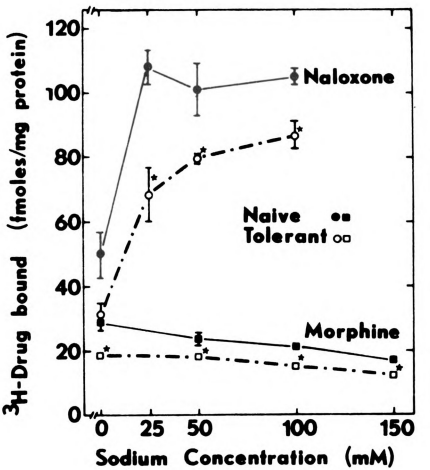


Figure 29. Chronic morphine treatment and the effect of sodium ion on binding of ^{3}H-morphine and ^{3}H-naloxone to brain slices.

Slices were incubated in 50 mM Tris-HCl buffer with the indicated sodium concentration. Concentration of both tritiated drugs was 3 nM. Animals were made tolerant by 72 hour morphine pellet implantation. Values are shown as mean $\pm$ S.E.M. of 6 (morphine) and 5 (naloxone) experiments. * indicates different from respective value for naive animals ($p<.05$).

concentrations. At 150 mM sodium, the ^{3}H-morphine binding was the same as nonsaturable binding in treated rats, whereas specific binding could be detected in naive rats. There was less binding to be inhibited by sodium in the tolerant group.

After chronic morphine treatment, the binding sites are still sensitive to the differential effects of sodium. However, it is not clear that morphine treatment enhances sensitivity of opiate binding sites, *in vitro*, to the differential effects of sodium.

Positive cooperativity between binding of ^{3}H-etorphine and non-labelled naloxone was found after chronic morphine treatment, in both incubation solutions (Figures 30 and 31). Therefore, it was not possible to determine the naloxone IC50 value for ^{3}H-etorphine (1 nM) binding. In Tris buffer, ^{3}H-etorphine binding tended to be less, following morphine treatment, at the lower naloxone concentrations (less than 6 nM), but the two curves did appear to converge above 12 nM naloxone. These are consistent with the reduction of binding affinity observed for the tolerant group in etorphine displacement experiments (Table 8). Binding of ^{3}H-etorphine, in CSF (Figure 31), at the lowest naloxone concentration, was greatly reduced in tolerant animals; at the next naloxone concentration ^{3}H-etorphine binding was only slightly less than control. These differences were observed in total ^{3}H-etorphine binding and when specific binding was expressed as percent of zero naloxone. This may be indicative of an increase of cooperativity among sites. However, this was not observed in etorphine displacement experiments in CSF (Table 7).

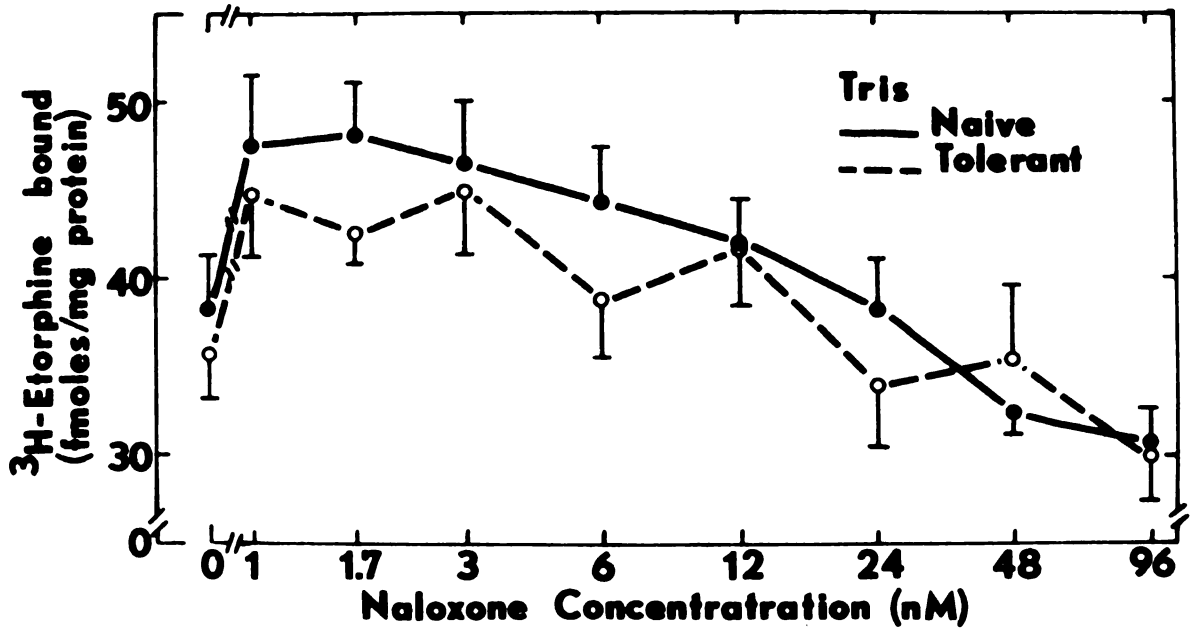


Figure 30. Effect of chronic morphine treatment on the interaction between naloxone and etorphine in Tris buffer.

Binding of ^{3}H-etorphine (1 nM) was determined at the concentrations of nonlabelled naloxone indicated. Values are shown as mean $\pm$ S.E.M. for 5 experiments. No significant differences between the two groups were found.

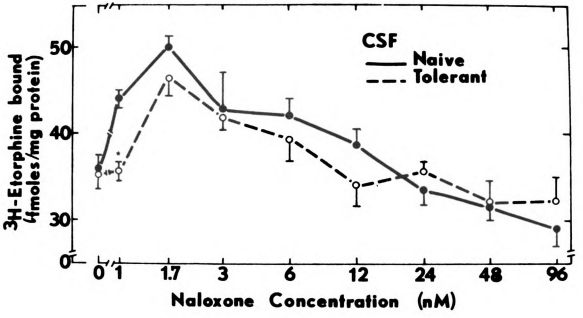


Figure 31. Effect of chronic morphine treatment on the interaction between naloxone and etorphine in artificial CSF.

Binding of ^{3}H-etorphine (1 nM) was determined at the concentrations of nonlabelled naloxone indicated. Values are shown as mean $\pm$ S.E.M. for 5 experiments. * indicates different from respective value for naive ($p<.05$).

DISCUSSION

Effects of Homogenization on Opiate Binding Sites

Saturable and specific binding, *in vitro*, of opiate agonists and antagonists is greater, in slices of brain tissue, than in homogenates. These differences may be explained by the uptake of opiate by an active transport process in brain slices. Scrafani and Hug (1968) have reported active uptake of narcotic analgesics into slices of rat cerebral cortex. Therefore, the possibility that the differences observed between slice and homogenate preparations, in the present studies, are related to active transport, rather than an analgesic receptor, must be considered. In the slice experiments, each slice was homogenized, individually, immediately after the binding reaction, so that any ^{3}H-drug which entered the cells (by active transport or passive diffusion) would be separated from the bound tritiated drug. The binding site could be a carrier protein, rather than receptor, and, *in vitro*, the two types of binding sites would have common characteristics, particularly saturability. However, Huang and Takemori (1976) were unable to demonstrate energy dependence of etorphine uptake into cerebral cortical or striatal slices, and they concluded that the saturable uptake observed was related to the putative receptor. Scrafani and Hug (1968), using much larger concentrations of narcotic, were able to inhibit only 20-30% of the uptake even with agents which classically block active processes (i.e., dinitrophenol,

iodoacetate, glucose deprivation, and nitrogen atmosphere). Therefore, it is not tenable to conclude that the differences observed between slice and homogenate preparations are related to an active transport process.

Alternatively, greater binding of opiates to brain slices may be explained by inhibition of the labelled drug binding in homogenates by an endogenous ligand. Enkephalin appears to be located within nerve cells, with a regional distribution similar to that of opiate binding sites (Elde et al., 1976; C. Pert et al., 1976; Simantov et al., 1976). Therefore, enkephalin would be included in both tissue preparations used. However, enkephalin may not have access to binding sites in the slice preparation (due to its localization within nerve cells). This would result in inhibition of the added, tritiated ligand in homogenates, but not in slices, and thus a reduction of apparent binding affinity, in homogenates. Such a reduction of binding affinity, in homogenates, was observed in the present studies, and initially was attributed to the presence of an endogenous ligand (Davis et al., 1975). However, Hambrook et al. (1976) have found that enkephalin is cleaved at the tyrosine-glycine amide bond by rat brain homogenates, and that this deactivation is quite rapid (10^{-7} M enkephalin was completely destroyed in less than 1 minute at 37°C by a brain homogenate with 3 mg protein/ml). While this explains the short duration of action, as noted earlier, it also rules out the possibility of competitive inhibition (in homogenate but not in slice preparations), over the 20 minute binding incubation period used in the present experiments.

Although competitive inhibition by enkephalin is unlikely, in the assay conditions used, the larger fragments of β-lipotropin could

be inhibiting binding of tritiated opiates. Activation of β-lipotropin, by aqueous extracts of brain homogenates, has been demonstrated (Ling and Guillemin, 1976), and it is possible that other, as yet unidentified, competitive inhibitors of opiate binding are released during homogenization. Conversely, when the tissue is homogenized in buffer, any activators required for binding would be diluted approximately 300-fold; this could reduce the apparent binding affinity, in homogenate, compared to brain slices. While there is not evidence to support or refute this possibility, co-factors are not generally required for drug-receptor binding.

Homogenization could also release hydrolytic enzymes from lysosomes. Enzymatic degradation of binding sites would result in a reduction of the concentration of available binding sites, and this was observed when homogenate and slice preparations were compared. Binding the sites with either agonist or antagonist protects the sites from inactivation by sulfhydryl reagents and detergents (Pasternak et al., 1975; Simon and Groth, 1975; Simon et al., 1975). Therefore, it is reasonable that binding sites in slices would be protected, by ligand, during the brief duration of homogenization and filtration used in the present slice studies. After pretreatment of animals with narcotic agonists or antagonists (for periods as short as five minutes), the concentration of binding sites, determined by *in vitro* binding studies, was increased (C. Pert et al., 1973; C. Pert and Snyder, 1976). These investigators concluded that the exogenous opiate, bound to the sites, protected these sites from enkephalin inhibition, and thus increased the number of sites available to tritiated drugs in the binding mixture. For this to be true, enkephalin or the opiate would have to bind irreverisbly, which has

not been observed; otherwise, the dissociation constant, rather than the binding site concentration, would be affected. It seems more likely that *in vivo* treatment with opiate essentially serves to prebind the sites and thus protect them, until the hydrolytic enzymes are removed during centrifugation. The increase in the concentration of binding sites observed, in the present studies, in tissue slices, as compared to homogenates, could be due to protection from hydrolytic enzymes by maintaining the normal subcellular compartmentalization of these enzymes.

Homogenization of the tissue would result in loss of normal ionic gradients. Both morphine and naloxone binding were less in brain homogenates. Therefore, a direct effect of sodium on the site is probably not involved. However, the concentration of potassium might be higher, at the receptor, in the homogenate, and thus reduce binding of both agonist and antagonist. Although this possibility was not tested, it seems unlikely, because in both preparations the tissue, and thus endogenous potassium, was diluted by a factor of 300. The ionic environment would be more physiological in brain tissue slices, particularly in the artificial CSF incubation media, than in homogenate preparations. However, in experiments comparing the slice and homogenate preparations, Tris buffer was used and thus the ionic environment was not physiological.

During homogenization, shearing forces are developed which break and otherwise physically disrupt cellular membranes and their components. The binding macromolecule may be directly affected, or the normal relationship among lipid and protein components lost. As the plasma membrane is broken apart, opiate binding sites may be lost, by cleavage of the site. The nerve membrane fragments close up, forming

vesicles. In these, the geometry of the binding site may be altered, and some of the binding sites may face inward (and thus be less accessible to the opiate in the media). The changes in cooperativity and the effects of sodium on binding strongly suggest that the binding site undergoes conformational changes; these may be restricted by alterations of the relationship between the membrane and the binding site. Simon et al. (1975a) were unable to bind their solubilized receptor, after allowing the ligand, which was bound before solubilization, to dissociate. It is not at all surprising, then, that homogenizing cells also reduces the capacity to bind opiates. Until the physical structure of the binding site is known, proposals as to the basis of this are speculative.

The concentration of binding sites, in homogenates, was found to be about one third that of slices. It is unlikely that such a large proportion of sites could be torn during homogenization. A more plausible hypothesis to account for the increased concentration of binding sites, in tissue slices, would be ligand protection from hydrolytic enzymes.

Cooperativity of binding was observed for both opiate agonists and antagonists in slice experiments. Such cooperativity was not apparent, even in displacement studies, which are very sensitive to interactions among sites, after homogenization. If, in the homogenate experiments, the concentration of ^{3}H-drug was greater than the dissociation constant, the initial increase of binding of ^{3}H-drug, at relatively low concentrations of nonlabelled drug, would not be observed, even if there were positive cooperativity among binding sites. The dissociation constant, estimated from morphine saturation studies, was 9.7 nM, whereas the concentration of ^{3}H-morphine used in

the displacement experiment was 3 nM. Therefore, the conditions of the displacement experiment were such that positive cooperativity among sites, if present, would have been detected. Other investigators have reported saturation curves, for both agonists and antagonists, which did not deviate from hyperbolicity (C. Pert and Snyder, 1973b, 1974; Simon et al., 1973; Wong and Horng, 1973; C.-Y. Lee et al., 1975) and have found Hill slopes ranging from slightly less than to slightly greater than 1 (Simantov and Snyder, 1976b). Simon et al. (1975c) reported Scatchard plots that were nonlinear and resembled the case of positive cooperativity (curved downwards at lower binding values). The Hill slope was later reported to be 1.5 (Simon, 1976). However, with this small degree of interaction, the deviation from linearity over the concentration range used is quite small and interaction among sites only slightly improved the fit of the data to the theoretical curve. If there were more than one population of binding sites, with different binding constants, the Scatchard plot of the saturable binding would be nonlinear, but would curve upwards at the lowest binding values (and two lines, each described by its own dissociation constant and binding site concentration, could be fitted to the data), opposite of the case for positive cooperativity. Downward concavity of a Scatchard plot could occur if the tritiated drug is bound less tightly than the non-labelled drug (Taylor, 1975). This is a problem where a radioactive label, such as ^{125}I, is added to the ligand, as for insulin. In opiate binding studies, tritium-labelled drugs are generally used, and the tritium replaces a hydrogen atom in the drug; therefore, it seems unlikely that the radiolabelled and nonlabelled drugs would have different binding affinities. In the displacement studies, if

the affinity for ^{3}H-drug was less than that for nonlabelled drug, the nonlabelled drug could displace ^{3}H-drug more readily, and thus the initial increase of ^{3}H-drug binding, at relatively low concentrations of nonlabelled drug, would not occur.

Results of the present experiments, as well as those reported by others, indicate that cooperativity among binding sites is retained poorly, if at all, following homogenization. Of the cellular mechanisms discussed above, the absence of cooperativity in homogenates could be most easily understood as a result of constraints on mobility and/or conformational changes of the binding sites within the cell membrane.

Cooperativity of receptor binding has been observed with other ligands. Acetylcholine binding to solubilized receptors exhibits positive cooperativity which is best described by the symmetry or two-state model (Gibson and Levin, 1977), similar to that proposed for the opiate receptor. Interactions among binding sites have been described for several peptide hormones, including insulin (negative cooperativity) and vasopressin (positive cooperativity) (see Kahn, 1976). Cooperativity has been shown for the glycine receptor using both electrophysiological and biochemical techniques (see Snyder and Bennett, 1976).

Analysis by the Hill equation (equation 1, page 26) is based on the assumption that interactions among sites are quite strong and, thus, at substrate concentrations greater than the dissociation constant, either none or all of the sites on a particular unit will be occupied. If the interactions are less strong, a significant number of units will be bound with an intermediate number of ligand molecules. In this case, n in the Hill equation underestimates the number of interacting sites. Therefore, the Hill coefficient (n_{app})

or slope (n_H) are best considered to be indices of cooperativity among sites, and not an accurate determination of the number of interacting sites (Segal, 1975). This is particularly important for treatments which may increase or decrease the extent of interaction.

Although opiate binding sites, in slices of brain tissue, appear to be a better model for the opiate receptor, *in vivo*, the slice preparation is not without its own disadvantages. The variability of the saturable or specific binding tends to be greater in slice studies. One replication of a given experiment generally will not yield a smooth saturation or displacement curve. Thus, binding constants should be estimated for the whole set of data, and the variability around the constants cannot be estimated. The binding site concentration varies among different brain areas (Hiller et al., 1973; Kuhar et al., 1973; C. Pert et al., 1976b) and, thus, at a given concentration of drug, the amount bound per mg of protein would vary between slices from the same brain. However, the brain areas used in the experiments were primarily in the moderate range of binding site density. Variability of nonsaturable binding was less than that for total and saturable binding.

To briefly summarize, in these studies homogenization resulted in a decrease of the binding site concentration and affinity measured *in vitro*. Furthermore, interaction among sites was observed only in slice experiments. In brain slices, the binding sites would be maintained in their normal state. Thus, the slice preparation is more relevant to the *in vivo* effects of narcotics. The positive interaction among binding sites is strong evidence for an oligomeric structure. The strength of this interaction is another binding

parameter which could be affected by chronic morphine treatment and the ionic environment *in vitro*.

Effects of Sodium on Opiate Binding Sites *in vitro*

Differential effects of sodium on opiate agonist and antagonist binding were observed in the present studies, using brain slices. In artificial CSF, with 150 mM sodium, the binding of etorphine was reduced and naloxone increased. The dissociation constants, determined from displacement analysis, for both the agonist and antagonist, were the same in Tris buffer (no sodium) and CSF. Therefore, the alterations of binding observed were due to differential alterations of accessibility to the binding site. For the agonist etorphine, the concentration of binding sites available in Tris buffer was found to be approximately twice that for artificial CSF, and the concentration of naloxone binding sites was greater in CSF than Tris buffer. The cooperativity among the sites was also differentially affected by sodium, and was found in CSF to be greater for etorphine but less for naloxone. Iso-osmotic sucrose or mannitol, or high concentrations of potassium (125 mM), did not mimic these differential effects of sodium.

Both Simon's and Snyder's groups have characterized the effect of sodium on opiate agonist and antagonist binding to membrane fractions isolated by low speed centrifugation. Simon et al. (1975b) found that the binding affinity is differentially altered by sodium ion. C. Pert and Snyder (1974) reported log drug-inhibition plots indicative of an effect on binding affinity. The model they currently use involves an alteration of affinity (Snyder and Bennett, 1976; Snyder, 1977). C.-Y. Lee et al. (1975) compared binding of naloxone and dihydromorphine in the absence of sodium (Tris buffer) and in

solutions with 47 and 150 mM sodium (simulated intracellular fluid and artificial CSF, respectively), using a particulate fraction of brain tissue (obtained by washing homogenates of brain at high speed). In their studies, sodium differentially affected both the concentration and affinity of binding sites. Thus, although sodium consistently inhibits agonist binding and enhances antagonist binding, the binding constants affected appear to depend on the type of tissue preparation used. As discussed above, homogenization itself affects the opiate binding site. Thus, it is not surprising that the effect of sodium ion on the binding site is different after different types of homogenization and centrifugation.

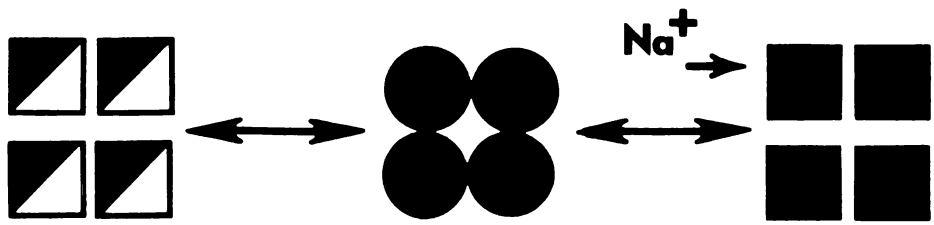
The maximum concentration of binding sites, in brain slices, available to naloxone (that is, detected in artificial CSF) is greater than the maximum available to etorphine (detected in Tris buffer). Thus, there are binding sites to which naloxone, but not etorphine, has access. This was also observed by C.-Y. Lee et al. (1975) for naloxone and dihydromorphine. The naloxone-etorphine interaction experiments (Figure 15) showed that nonlabelled naloxone has access to at least a portion of the ^{3}H-etorphine sites (otherwise nonlabelled naloxone would not have affected ^{3}H-etorphine binding). The amount of ^{3}H-etorphine specifically bound is significantly greater in Tris buffer, compared to artificial CSF, at higher concentrations of nonlabelled naloxone (12-96 nM). When the concentration of nonlabelled naloxone is 50-100 times greater than that of ^{3}H-etorphine (and also 6-25 times greater than the etorphine dissociation constant), and if naloxone has access to all etorphine binding sites, then the amount of ^{3}H-etorphine bound would be the same in both Tris buffer and artificial CSF, and the two binding curves would meet. However,

^{3}H-etorphine binding was significantly less in artificial CSF, at higher naloxone concentrations. Therefore, in Tris buffer, there are etorphine binding sites which naloxone does not have access to, or etorphine specific sites. Such agonist specific sites have not been observed by others. However, such sites would not be obvious in saturation experiments. Furthermore, the agonist specific sites would not be observed in competition displacement experiments previously reported (C. Pert et al., 1973; C. Pert and Snyder, 1974) because binding of an antagonist, ^{3}H-naloxone, was measured.

The first model postulated to explain the differential effect of sodium involved a conformational change of the site, with sodium as an allosteric effector for this change (C. Pert and Snyder, 1974; Simon et al., 1975b). Simon et al. (1975c) noted that this model would predict cooperativity among binding sites and, as already discussed, concluded that the nonlinearity of Scatchard plots of their saturable binding data was due to positive cooperativity among binding sites. They then suggested a modification of the allosteric model to include cooperativity (Simon et al., 1975c; Simon, 1976). According to the revised model, the binding site exists as a monomer and dimer in the absence of sodium, but only the dimer can convert to the sodium dependent conformation. Thus, in high sodium concentration (100 mM), the agonist binding observed would be to monomers. The results of the present experiments, with brain slices, are not consistent with this model. The degree of cooperativity of etorphine binding was found to be greater, in the presence of sodium, indicating binding of agonist to an oligomeric, rather than monomeric, form; similarly, cooperativity of naloxone binding was less in artificial CSF than Tris buffer, indicating that a smaller proportion of the

sites available to naloxone, in the presence of sodium, are in an oligomeric state. Furthermore, the number of sites, rather than their binding affinity, was differentially affected by sodium ion in the present slice studies.

These data can be explained by a modification of Simon's allosteric model for the opiate receptor (Simon, 1976). Sodium differentially affects two binding parameters, cooperativity and concentration of binding sites. The opiate binding site could exist in three states: a conformation which can bind both agonist and antagonist, an agonist-specific form, and an antagonist-specific form. Some of the specific conformations are interconvertible, under the proper conditions. However, a portion of the specific sites are locked into their respective conformations. The shared conformation has the greatest degree of binding site cooperativity, and the interaction is weaker for the two restricted forms. Sodium shifts the equilibrium in favor of the antagonist-only form. Thus, the number of sites for agonists decreases, and more of these take the shared conformation (and thus cooperativity increases). The binding site symbols are shown partially



separated to indicate reduced cooperativity; this does not necessarily mean that binding sites physically associate or dissociate (this may be the case, though). In this model, the conformational change induced by sodium involves altering agonist/antagonist accessibility and cooperativity, and not affinity, and thus is different from models

developed for homogenate experiments (Simon et al., 1973; Simon and Groth, 1975; C. Pert and Snyder, 1974). However, for the agonist and antagonist used in these studies, the binding site concentration and cooperativity and not affinity were affected by sodium.

The model presented here predicts that opiate agonists, antagonists and mixed agonists/antagonists interact with the receptor differently. Martin (1967) postulated that agonists and partial agonists interact with different analgesic receptors, and that partial agonists also interact with the agonist site, but have no effect after binding. Thus, the partial agonists function as competitive inhibitors at the agonist site. Smits and Takemori (1970) reported that the pA_2 values for naloxone antagonism are different for narcotic agonists and mixed agonists/antagonists, and concluded that either the two classes interact with two different receptors or that they interact with the same receptor, but in a different manner. Similarly, enkephalin and enkephalin amide were found to have smaller pA_2 values than normorphine, indicating that the receptor for the peptides has a lesser affinity for naloxone than the normorphine receptor (Frederickson et al., 1976). Martin et al. (1976) determined the effects of narcotic drugs (agonists and mixed agonists/antagonists) on six parameters in chronic spinal dogs (measured were pain, by the flexor reflex, skin twitch to thermal stimulation, pupil diameter, body temperature and respiratory and heart rates). The data obtained are best explained on the basis of different effects at three types of opiate receptors which, though closely related stereochemically, do differ in their effects. The μ-receptor, which binds morphine-like drugs, produces general depression of nociceptive reflexes and indifference, the K-receptor, which binds ketocyclazocine-like drugs, produces miosis and sedation, and

σ-receptor, which binds SKF 10,047-like drugs, produces mania and delirium. The three sites, as thus far characterized, are quite similar to the conformations hypothesized for binding sites in brain slices and, as such, provide further evidence that opiate binding to tissue slices is a more appropriate model, *in vitro*, for the receptor *in vivo*.

Terenius and Wahlstrom (1976) have reported that *in vitro* binding of opiate agonists and antagonists does exhibit heterogeneity. Inhibition of binding of one ^{3}H-drug by nonlabelled drug was plotted against inhibition of a second ^{3}H-drug by the same competitor. At a given concentration of competitor, in the two binding assays, the chemical concentration of both drugs was the same; the only difference was which drug was tritiated. If the binding sites for the two drugs are identical, then the inhibition vs inhibition plot should have a slope of 1. This was the case for some opiate drug combinations, but not others. They concluded that agonists are limited to one type of receptor, but that antagonists can bind to this plus an additional site. Although they did not observe agonist specific sites (perhaps because they used synaptic plasma membranes rather than tissue slices), they did find evidence for heterogeneity of sites, similar to that of the proposed three state model. This type of analysis is not based on the law of mass action, and thus binding constants are not determined; the only information this analysis provides is whether the nonlabelled inhibitor competes with the two radiolabelled drugs in the same manner. Thus, many combinations of inhibitors and radiolabelled indicators must be used to characterize heterogeneous sites.

In vitro, sodium ion acts as a heterotropic activator or inhibitor (that is, enhancing or inhibiting the binding of a molecule other

than itself), depending on the agonist/antagonist properties of the drug. Electrophysiological studies of narcotic action suggest that the movement of sodium ion *in vivo* is differentially affected by agonists and antagonists. Zieglgansberger and Bayerl (1976) observed that opiates (either morphine or fentanyl, administered microiontophoretically or systemically, respectively) blocked the increase in firing rate of dorsal horn neurons in response to noxious stimuli. The same stereo- and chemical-specificity, demonstrated *in vivo* and for binding *in vitro*, was observed. Levorphanol, but not its inactive enantiomorph dextrorphan, depressed firing, and this was reversible by naloxone (given either directly or systemically), and was not accompanied by hyperpolarization of the cell membrane. Morphine blocked L-glutamate-induced depolarization and this was reversed by naloxone. Therefore, it was concluded that opiate agonists impair sodium influx and that this is mediated by a specific opiate receptor on the postsynaptic membrane. Opiate agonists prevent the increase in sodium conductance essential for impulse propagation, whereas antagonists have no effect on sodium conductance. It is tempting to speculate that *in vivo* agonists and antagonists act as heterotropic activators or inhibitors, respectively, of sodium. Thus, in CSF, binding of an agonist (to the shared conformation of the receptor) shifts the equilibrium away from the low cooperativity antagonist-only form. This conformational shift of the receptor, then, would be accompanied by a decrease of sodium conductance, perhaps by physically altering nearby sodium channels.

Following 72 hour morphine treatment, morphine binding, to brain slices, was found to be reduced, and this was attributable to a decrease of the binding affinity. The results of initial saturation

experiments were quite similar to the inhibitory effect of sodium ion on agonist binding reported by other investigators (C. Pert and Snyder, 1974; Simon et al., 1975; C.-Y. Lee et al., 1975), and in both cases the reduction of binding was attributable to a decrease of the binding affinity. It was hypothesized that the effects of chronic morphine treatment are mediated by an increase of the binding sites' sensitivity to sodium's effects. Although the differential effects of sodium on the opiate binding site, in brain slices, were observed following chronic morphine treatment, the results of the present studies do not support a causal or primary role for sodium in tolerance development. In the slice studies, the sodium-induced enhancement of naloxone binding and inhibition of etorphine binding were apparently due to a change of the binding site such that accessibility of agonist or antagonist to the site is decreased or increased, respectively; and the dissociation constants for the two ligands were not affected. The cooperativity among binding sites was also found to be affected by sodium; the degree of interaction was less for antagonists and greater for agonists, in a high sodium environment. In contrast, the concentration of etorphine binding sites was unaffected by chronic morphine treatment and the binding affinity was reduced twofold. The cooperativity index was less in the tolerant animals (Table 7). The etorphine binding parameters altered by sodium ion and 72 hour morphine treatment were not the same. If chronic morphine treatment mimics the effects of sodium on naloxone binding, then, in Tris buffer, binding would be relatively unaffected and, in artificial CSF, binding would be greater, following morphine treatment. The opposite results were observed. In tolerant animals less naloxone was bound in Tris buffer, whereas in artificial CSF binding was the

same in the control and treated groups. In Tris buffer the concentration of naloxone binding sites was the same following morphine treatment, while the dissociation constant was increased (binding affinity decreased) and the index of cooperativity was decreased; in contrast, sodium-induced enhancement is due to an increase in the concentration of sites available to naloxone, and not to an effect on binding affinity. Also, the magnitude of the stimulation of naloxone binding and inhibition of morphine binding, by increasing concentrations of sodium, did not appear to be increased following chronic morphine treatment. On the basis of these results, it must be concluded that increased sensitivity to the differential effects of sodium ion is not the mechanism of tolerance development. The effect of chronic morphine treatment on naloxone binding (measured in Tris buffer) were the same as those found for etorphine binding (in Tris buffer). This suggests that the binding sites affected by chronic exposure to morphine are the agonist and mixed (agonist plus antagonist) types postulated earlier, and that the reduction of binding affinity will be observed for any ligand at the site. Martin and co-workers (1976) have shown that tolerance to morphine is mediated by μ receptors and Martin et al. (1974) demonstrated that naloxone is a competitive antagonist at the μ receptor. The effect of chronic morphine treatment to reduce opiate binding appears to be a direct effect on the receptor itself, and not an effect mediated by sodium.

Effects of Chronic Morphine Treatment on Binding Sites *in vitro*

Chronic treatment with morphine is associated with a reduction of saturable or specific opiate binding, to slices of brain. In the present studies, binding of ^{3}H-morphine to homogenates was not

affected by *in vivo* treatment with morphine. This is in good agreement with previous reports, in which no effects of *in vivo* opiate treatment, on the *in vitro* opiate binding site, which correlate with tolerance development have been found (Klee and Strety, 1973; C.-Y. Lee et al., 1973; C. Pert et al., 1973; Hitzemann et al., 1974; C. Pert and Snyder, 1976). In slice studies, nonsaturable ^{3}H-morphine binding (in the presence of 10^{-5} M nonlabelled morphine) was unaffected by 72 hour morphine treatment. However, total, and therefore saturable, ^{3}H-morphine binding were less in the morphine treated group. Graphic analyses of saturation experiments consistently indicated a reduction of binding affinity, *in vitro*. Alterations of the binding site, by homogenization, blocks the expression of the effect of chronic morphine treatment. This is consistent with the suggestion, discussed previously, that perturbations of the binding site and surrounding membrane, during homogenization, restrict mobility and fluidity, or otherwise adversely affect binding. It is unlikely that enkephalin inhibition occurs in the homogenate preparation; however, if so and if Simantov's and Snyder's (1976c) enkephalin-mediated theory of tolerance is correct, effects of chronic morphine treatment would be seen in the homogenate, and not in tissue slices. Ligand protection of binding sites from degradation, as already discussed, may account for the increase of binding site concentration in slices. If the sites affected by chronic morphine treatment are more sensitive to endogenous hydrolytic enzymes, then these sites might be available in slices, but not homogenates (because of subcellular separation of the binding site and enzymes).

Although it is not yet possible to select between these hypotheses, the ability to detect an effect of chronic morphine treatment,

consistent with tolerance development, is further evidence that the binding site, in slice, is a superior *in vitro* model for the opiate receptor.

In displacement experiments, the nonsaturable binding of ^{3}H-morphine to brain slices was not affected by chronic morphine treatments, consistent with saturation studies. The total amount of ^{3}H-morphine bound was reduced (at lower concentrations of nonlabelled morphine, Figure 22). The decrease of binding observed for different treatment and withdrawal times correlates well with the degree of tolerance observed after these treatments. This is further evidence that the alterations of binding *in vitro* are related to tolerance development. Binding was also reduced in the group treated with morphine for 3 weeks, by subcutaneous injection. Exposure to morphine by 2 different administration techniques is associated with decreased ability of binding sites, *in vitro,* to interact with morphine. This reduction is reversible if morphine treatment is terminated, but otherwise will be maintained for up to 3 weeks, if morphine treatment is continued.

After *in vivo* treatment with morphine, some morphine may remain in the brain tissue and would competitively inhibit binding of tritiated opiates *in vitro* (and thus could account for the decreased apparent affinity in saturation experiments). In displacement experiments, this would be the same as adding nonlabelled morphine to the incubation mixture, without adjusting the concentration on the abscissa, which would, in Figure 21, result in a parallel shift of the binding curve to the left. Since a parallel shift was not observed, it may be concluded that the reduction of binding observed is due to a change at the binding site, and not to residual morphine.

Furthermore, if inhibition by residual morphine was the cause, reduction of binding should also be observed in tissue homogenates and should not be observed in slice displacement experiments, 1 and 4 days after removal of the pellets (Figure 22).

In displacement experiments, the binding constants (determined by nonlinear regression analysis of the data) affected by chronic morphine treatment were different for morphine (Table 6) and etorphine (Table 7). For morphine, the concentration of binding sites was decreased and the strength of interaction among sites increased, whereas for etorphine the binding affinity and cooperativity were reduced following chronic morphine treatment. While it could be argued that this reflects intrinsic differences between the two drugs (in homogenates the sodium response ratio for etorphine is considerably less than that for morphine, suggesting etorphine may be less pure in its agonist properties (C. Pert et al., 1973) this seems unlikely. Results of naloxone binding experiments seem to indicate that the reduction of binding affinity and cooperativity, following chronic morphine treatment, are determined by the conformation of the site, and are relatively independent of the nature of the ligand. More likely these conflicting results mean that the displacement analysis is too sensitive to biological variation within the data. Therefore, although the decreases in agonist binding are observed, it is difficult to determine which of the binding parameters are altered by chronic morphine treatment.

After treatment with morphine, tolerance to the analgesic effects of morphine is evident from the parallel shift of the log dose response curves (Figures 15 and 16) and concomitant increase of the dose required to produce a given degree of analgesia (Table 4). This

has been demonstrated with the present treatment regimen as well as by others (Way et al., 1969; Smits and Takemori, 1970). Such a parallel shift indicates a reduction of apparent binding affinity and can result from competitive inhibition at the receptor (as would be the case for the shift observed following naloxone pretreatment, Takemori et al., 1970) or an alteration of the receptor such that binding affinity is less in tolerant animals. In the analgesia assessment experiments reported here, the animals were withdrawn for at least 6 hours prior to testing, by which time the concentration of residual morphine would be quite low (Dahlstrom and Paalzow, 1976; Hipps et al., 1976). The present analgesia experiments and the bulk of the slice binding experiments support the conclusion that tolerance to narcotics is accompanied by, and may be the result of, decreased affinity of the analgesic receptor for opiate agonists.

After chronic morphine treatment direct application of morphine (Satoh et al., 1976) or enkephalin (Zieglgansberger et al., 1976) did not depress firing of cells in the sensorimotor cortex (which are sensitive to opiate agonists in naive animals). These studies strongly support the hypothesis that opiate tolerance is mediated, at least in part, by alterations of the receptor. However, these studies did not distinguish between a reduction of the drug-receptor interaction and inability to transduce the physical event of interaction into a physiological response.

Individuals tolerant to and dependent on opiates seem to be more sensitive to narcotic antagonists (see Way et al., 1969) and *in vivo* studies, using the pA_2 method, demonstrated an increase of the apparent affinity, for narcotic antagonists, after exposure to opiate agonists (Takemori et al., 1973; Tulunay and Takemori,

1974a,b). Increased naloxone efficacy in tolerant animals would be reflected in the naloxone-etorphine interaction experiments as greater inhibition of ^{3}H-etorphine binding by nonlabelled naloxone. Although ^{3}H-etorphine binding did appear to be slightly less in tolerant animals, specific binding, as percent of zero naloxone, was the same in both naive and tolerant animals. Thus, the reduction observed can be explained by a reduction of etorphine binding per se, rather than an increase in competitiveness of the antagonist for the agonist binding sites, or increased affinity for the antagonist. The efficacy of naloxone is also increased by treatments which elevate serum concentration of corticoids, such as ACTH, dexamethasone and corticosterone (Harris et al., 1976). Stress, associated with opiate withdrawal, appears to play a major role in the increase of naloxone efficacy induced by chronic exposure to morphine, rather than a specific receptor mechanism. In agreement with this, the binding affinity for naloxone was not found to be increased in tolerant animals.

In summary, the present studies have demonstrated that tolerance to morphine is accompanied by a reduction of opiate agonist binding, *in vitro*. These results are most readily attributable to a reduction of binding affinity, and do not appear to be related to the effects of sodium on binding. The decrease of binding was observed only in tissue slices, and not in homogenate preparations. This further strengthens the conclusion that the slice preparation is a better *in vitro* model for the opiate receptor.

Theories of Opiate Tolerance

Immune mechanisms. Tolerance has been found to persist as long as 15 months after morphine treatment was terminated (Cochin and Kornetsky, 1964), which suggests a "memory" such as could occur if the immune system was involved in tolerance development. This could explain the observed parallel shift of the dose-response curve, because the immune protein would compete with the receptor for the test dose, and the apparent requirement for protein synthesis. If the immune protein were close to morphine's site of action, this could account for the observed insensitivity to microiontophoretically applied morphine (Frederickson and Norris, 1976; Satoh et al., 1976; Hill, 1976). However, passive transfer of tolerance, via an immune protein, has not been successful (Kiplinger and Clift, 1964; Smits and Takemori, 1968). Furthermore, tolerance to morphine can be detected within 48 hours after one injection of morphine (Huidobro et al., 1976) and, in the present studies, was observed after 72 hours of morphine treatment. These are inconsistent with synthesis of a specific anti-morphine protein by the immune system (see Eisen, 1974). If a morphine binding protein, synthesized under control of the immune system, is responsible for morphine tolerance, by competitive binding, then more binding sites would be available to tritiated agonists *in vitro*. In the present studies, the effect observed was a decrease of agonist binding. There is, then, no evidence for an immunologic tolerance mechanism.

Neuromodulators. The involvement of various neurotransmitters in the acute and chronic effects of morphine and the effects of

morphine administration on neurotransmitter systems have been studied (see Way, 1972; Takemori, 1974). Acetylcholine, norepinephrine and dopamine appear to mediate effects of acute administration and withdrawal, but not tolerance devlopment. Alterations of the serotoninergic system, consistent with tolerance development, have been reported, although some of the results have not been replicated (see Way, 1972). Causal involvement of any one neurotransmitter system in the development of tolerance has not been conclusively demonstrated. The regional distribution of opiate binding does not correlate to that of one neurotransmitter system, and given the wide range of narcotic effects mediated by the central nervous system (and apparently via different neurotransmitter systems), and to which tolerance develops, it is perhaps not reasonable to try to attribute all the observed effects to perturbations of only one neurotransmitter system.

The identification of an endogenous ligand for the opiate receptor, with characteristics of a neuromodulator, led to speculation that tolerance may be mediated by alterations of this system. Simantov and Snyder (1976c) found increased amounts of enkephalin in brain tissue following morphine treatment (5 days), and Clouet and Ratner (1976) reported preliminary evidence of decreased enkephalin turnover. Simantov and Snyder (1976c) hypothesized that morphine inhibits enkephalin release by feedback inhibition and that, since enkephalin is not released, more morphine is required to replace enkephalin at the receptor. According to this model, the amount of morphine required to produce an equal degree of analgesia is greater in tolerant animals because enough morphine to replace enkephalin, which would normally be present, must be given, in addition to the test dose. The effect of adding a constant amount of morphine (to

replace enkephalin) to increasing test doses would, on a log dose-response curve, be an increase in slope. However, the log dose-response curves for treated animals are parallel to those of control groups (Figures 16 and 17). Thus, this enkephalin mediated theory predicts changes contrary to what has been observed, so it must be concluded that this theory is insufficient to explain development of tolerance. Furthermore, this theory is predicated on the hypothesis that enkaphalin is a mediator in a system which is normally active and which is involved in nociception. Until the roles of enkephalin in the nervous system are known, such a hypothesis is untenable. The evidence indicates that the functioning of an "enkephalinergic" system is affected by chronic morphine treatment. However, this change does not appear to be causally involved in tolerance development.

Redundancy theory. According to this hypothesis, there are at least two (i.e., redundant) pathways for each centrally mediated effect of the narcotics. One of these is inhibited by opiate. The other, under continual influence of opiate, hypertrophies functionally, and is able to substitute for the inhibited pathway, so that normal functioning of the system is restored. This is tolerance. When drug is no longer present, the first pathway is no longer inhibited, and becomes active. The second, hypertrophied pathway is also functioning; thus, the total activity, for the particular system, is increased. This is the rebound excitation observed during withdrawal. Depending on the relative degrees of inhibition and hypertrophy, greater or lesser acute, tolerant and abstinence effects will be seen (see C.-Y. Lee and Akera, 1975). This hypothesis is attractive because it readily accounts for different sensitivity of different

systems to the effects of narcotics. However, it is quite difficult to test. The changes predicted by this would most readily be observed as changes of neurotransmitter metabolism, and this would not necessarily be limited to one neurotransmitter system. Thus, the redundancy theory may account for the alterations observed in different neurotransmitter systems. The redundancy theory does not postulate changes of the opiate receptor and thus the present results are in apparent conflict with this theory. It should be noted that the redundancy theory and the decreased receptor affinity theory are not mutually exclusive.

Receptor mediated mechanisms. The results of the present studies indicate that tolerance to narcotics is more easily explained as a direct effect on the receptor, to decrease the affinity of the receptor for opiate agonists. The acute effects of morphine appear to be mediated by a specific opiate receptor, through different neurotransmitter systems (see Way, 1972). That is, the interaction of an opiate agonist with its receptor causes functional changes of other neural systems, the end result of which are the pharmacologic effects of the opiates. If, as a result of chronic opiate treatment, the ability of the opiate receptor to interact with opiate agonists is decreased, there would be less of an effect on other systems and thus pharmacologic tolerance would be apparent. According to this scheme, alterations of effects on synthesis and degradation of the various neurotransmitters would be secondary effects, determined by normal control mechanisms for the particular system, and thus it might not be possible to correlate aberrations of any one system (particularly

if whole brain turnover or concentration is determined), consistent with tolerance development.

Narcotic antagonists do not induce tolerance (Jasinski and Martin, 1967) and, when administered concomitantly, can block the development of tolerance to repeated administration of agonists (Orahovats, 1953; Lomax and Kirkpatrick, 1967). These results indicate that the mechanism for tolerance development can discriminate between active and inactive ligands. This capacity has been demonstrated for the receptor in studies using microiontophoretic application of morphine and/or naloxone to nerve cells described earlier (Zieglgansberger and Bayerl, 1976).

Two variations of receptor mechanisms are commonly considered: synthesis of inactive, or silent, receptors, and decreased binding affinity of active receptors. If silent receptors are synthesized, they could competitively inhibit binding of ligand to active receptors. Both postulates are consistent with observed tolerance to microiontophoretically applied morphine and the parallel shift of the log dose-response curve. The effect of silent receptors on *in vitro* binding would be an increase of binding in tolerant animals, due to an increased number of binding sites available. In the present studies a reduction of binding of tritiated agonists, associated with a reduction of binding affinity, was observed in slice experiments. Therefore, the present studies contradict the silent receptor postulate and provide evidence that receptor affinity, for narcotic agonists, is reduced during chronic morphine treatment.

How this comes about is speculative. Tolerance to morphine develops over time, perhaps because old receptors are being replaced with new receptors which, because of the presence of morphine, are

somewhat different, in that their agonist binding affinity is reduced. Conversely, after treatment is terminated, tolerance is lost, as altered receptors are replaced by normal receptors. Recent work, in Loh's laboratory, has demonstrated that phosphorylation of non-histone proteins and chromatin template activity (from oligodendroglial nuclei) are both increased following chronic morphine treatment (N. M. Lee et al., 1975; Oguri et al., 1976). These are both indicative of an increase of DNA transcription in the tolerant state. Tolerance development can be blocked by inhibitors of protein synthesis (Cox and Osman, 1970), perhaps because receptor turnover is also blocked. In cultured rat diaphragm, loss of acetylcholine receptors (after denervation) was blocked by protein synthesis inhibitors (Berg and Hall, 1974). To prove this hypothesis, it would be necessary to demonstrate turnover of the receptor, within the time course of tolerance development. The present time course studies are consistent with, but do not prove, this theory. An irreversibly bound ligand would greatly facilitate this.

Further Considerations

In the present studies on opiate binding, cooperativity of binding has been demonstrated in brain slice, but not homogenate, preparations. In a physiological incubation medium, the apparent degree of cooperativity was found to be greater for the agonist etorphine than for the antagonist naloxone. This may be indicative of the fundamental difference, between agonist and antagonist interactions with the receptor, which determines whether the molecule will have agonist or antagonist activity. Further studies, with more agonists and antagonists, are necessary to support this hypothesis.

Loh and his co-workers have presented evidence which strongly suggests a functional role for cerebroside sulfate within the opiate receptor. It would be interesting to determine what the role of cerebroside sulfate is and whether the observed effects of chronic morphine treatment on binding are related to a cerebroside sulfate moiety of the receptor.

The present studies have been concerned with the drug-receptor interaction. *In vitro* studies on neuroblastoma X glioma hybrid cells have shown that agonists (morphine and enkephalin) inhibit adenylate cyclase initially but, as exposure to morphine is continued, the specific activity of the enzyme increases and the concentration of cyclic AMP in the cells returns to normal. Upon removal of morphine, and thus removal of inhibition, the cyclic AMP concentration of the cell increases (Klee and Nirenberg, 1976; Sharma et al., 1975a,b). Because cultured cells were used, the relevance of this is questionable. However, other investigators have demonstrated that treatments which increase cyclic AMP concentrations increase the intensity of withdrawal symptoms (Collier and Francis, 1975). This suggests that adenylate cyclase may be one receptor for the narcotic agonists and it would be interesting to determine the relationship between inhibition of adenylate cyclase activity and reduction of binding in narcotic tolerance.

SUMMARY

Differences of opiate binding *in vitro* have been found between slices and homogenates of brain tissue. In the slice, concentration of binding sites and their affinity for ligand are greater. More important, these sites exhibit positive cooperativity and are sensitive to chronic exposure to morphine. In tissue homogenates neither interactions among binding sites nor alterations of binding after tolerance development were observed.

Positive cooperativity among opiate binding sites is predicted by the allosteric model for sodium effects on the receptor but had not yet been convincingly demonstrated. In slice the differential effects of sodium on the opiate binding site are seen and the degree of cooperativity is affected by sodium. The concentration of binding sites for agonists and antagonists is also differentially affected by sodium. The results of slice experiments are consistent with a 3 state model for the receptor, with differing degrees of cooperativity and specificity for opiate agonists and antagonists. This effect is specific for sodium and is not a result of alterations of ionic or osmotic strength.

Chronic treatment with morphine and development and offset of tolerance are accompanied by alterations of opiate agonist binding. Inhibition of morphine binding correlates well with the degree of analgesic tolerance developed or remaining following chronic treatment. Etorphine binding was also less after chronic morphine treatment.

When measured in CSF naloxone binding was unaffected by exposure to morphine; however, in Tris buffer, which would favor the agonist conformation of the binding site, naloxone binding was reduced. The data indicate that the drug-opiate agonist site interaction is blocked or inhibited during tolerance. The binding constants were not consistently altered and thus the mechanism by which the inhibition of binding develops is not well determined. Many of the *in vitro* binding studies indicate that binding affinity is reduced. This is in agreement with the parallel shift of the log dose-analgesic response curves following chronic morphine treatment and with the insensitivity of neural cells to morphine-induced inhibition of firing after 5 day morphine treatment.

Thus, in the present studies an opiate binding site has been found in brain slices *in vitro* which exhibits positive cooperativity and which is sensitive to the effects of chronic morphine treatment. Previous *in vitro* binding studies have not successfully demonstrated these properties because the binding sites are altered during preparation of the tissue. These studies provide additional evidence that tolerance to narcotic drugs is mediated, at least in part, by a reduction of the ability of the receptor to interact with the narcotic agonists.

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